Doctoral Dissertation

Development of Systematic Evolution of Ligands by Competitive enrichment-SELCO for highly selective and multiplex *in vitro* selection of DNA aptamers

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Thesis Title	Development of Systematic Evolution of Ligands by Competitive enrichment-SELCO for highly selective and multiplex *in vitro* selection of DNA aptamers
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Abstract

Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule and can be engineered through *in vitro* selection equivalently known as SELEX (systematic evolution of ligands by exponential enrichment). The technique for selection was developed in 1990 and since then the field of molecular selection has seen continuous evolution due to the active interest of biologists. Molecular recognition, an interesting characteristic of aptamers allows them to rival antibodies popular for their diagnostic property. In addition to this, aptamers have far-reaching applications in therapeutics, bioimaging, drug-discovery and other. However, in addition to high affinity, specific recognition holds more essential significance for the reliable detection of target molecules in the presence of other similar configuration bulk molecules. Despite the enormous number of reports on aptamer, a small number of aptamers have reached clinical trials stage. This limitation can be met through an alternative for selection of highly specific candidates to a certain extent. In addition, the traditional method of SELEX is still complex, time consuming and high cost. The selection process involves multiple rounds of amplification leading to undesirable PCR bias and the successful rates are low in general. Here, we present a novel approach called 'Competitive non-SELEX' and termed as 'SELCO' (Systematic Evolution of Ligands by Competitive enrichment) for readily obtaining aptamers that can discriminate between highly similar targets. This approach is based on the theoretical background presented here, in which under the co-presence of two similar targets, a specific binding type can be enriched more than a nonspecifically binding one during repetitive steps of partitioning with no PCR amplification between them. This principle was experimentally confirmed by the selection experiment for influenza virus subtype-specific DNA aptamers. Namely, the selection products (pools of DNA aptamers) obtained by SELCO were subjected to a DEPSOR-mode electrochemical sensor, enabling the method to select subtype-specific aptamer pools. From the clonal analysis of these pools, only a few rounds of *in vitro* selection were sufficient to achieve the surprisingly rapid enrichment of a small number of aptamers with high selectivity, which could be attributed to the SELCO principle and the given selection pressure program. The subtype-specific aptamers obtained in this manner had a high affinity (e.g., K_D = 82 pM for H1N1; 88 pM for H3N2) and negligible cross-reactivity. The kinetics of H1N1-specific DNA aptamer showed close resemblance with respective monoclonal antibody thus suggestive of aptamer potential comparable with antibodies. By making the H1N1-specific DNA aptamer a sensor unit of the DEPSOR electrochemical detector, an influenza virus subtype-specific and portable detector was readily constructed, indicating how close it is to the field application goal. The identification and evaluation of the selected aptamers were performed by cloning and sequencing. The identified aptamers were tested for their kinetic parameters by SPR and their specificity was evaluated by electrochemical detection. So, a novel technology for isolation of specific aptamers for multiple targets has been described here. Rapid, PCR-free and simple concept along with the compliance of experimental inferences with the theoretical explanation of SELCO holds significance in the future potential of this technology for other *in vitro* selection.

Keywords: Aptamer, SELEX, SPR, electrochemical sensor, influenza virus

Preface

The theory of natural selection and evolution has been determining in understanding the idea of origin of life. Several theories were proposed to understand the concept of how life started from the beginning. The discovery of RNA as a self-replicating entity marked the potential of nucleic acid molecule to be over and above simply units of genetic information. Furthermore, the assembly of different processes made possible to amplify and select molecules *in vitro*. In molecular biology, a combinatorial chemistry technique for producing oligonucleotide ligands (aptamers) that bind target molecules is *in vitro* selection also referred as Systematic Evolution of Ligands by EXponential enrichment (SELEX). The fundamental of *in vitro* selection is to isolate binding ligands from non-binding ligands for a target molecule by repeated selection cycles of binding, partitioning and amplification. Aptamers have been widely applied in analytical, bioanalytical, imaging, diagnostic and therapeutic fields. Due to the inherit merits of this technology, substantial achievements have been made regarding selections, modifications and applications of aptamers. However, few aptamer-based products have successfully entered into the clinical and industrial use. Besides, it is still a challenge to obtain highly specific aptamers in an efficient manner. This study was designed with the mindset to be able to select specific aptamers for real time point-of-care application. The selection forces administered during the process are determining in the selectivity of isolated ligands. On theoretical background, the introduction of competition induced selection force with the presence of a competitor target is a novel approach nearer to specific aptamer selection. The preliminary findings during the course of this research are supportive for the development of a unique technique of Systematic Evolution of Ligands by COmpetitive enrichment (SELCO). Also, simple and straightforward evaluation can be attained by the integration of the selected aptamer with electrochemical-sensing platform. This research has been developed and advanced under the supervision of Prof. Yuzuru Takamura and Assoc. Prof. Manish Biyani at School of Material Science in Japan Advanced Institute of Science and Technology. I hereby declare that details furnished here are true and correct to the best of my knowledge and belief and I shall be held liable for any misleading/untrue information.

Ankita Kushwaha

Acknowledgement

This research has been accomplished with support of particular individuals. The guidance of Prof. Yuzuru Takamura has been extremely helpful and of utmost importance in the successful completion of this research. Also, dialogue and consultation with him helped develop insight, learning and critical thinking skills essential for scientific research. The guidance of Assoc. Prof. Manish Biyani have principal role in the development of this research from scratch to concrete. His continuous mentoring by innumerable discussions on day-to-day observations have far-reaching significance in the progression of this work. The enlightenment developed from the theoretical perspective for this research has been the contribution of Prof. Koichi Nishigaki. The experienced and trusted guidance of co-worker Keiko Kawai have been absolutely essential for developing hands-on training skills for careful equipment handling. Also, the comments of the committee members have sincerely been considered and proven vital for the improvement of this research. The indefinite love, continuing support of parents and their lessons of right-minded approach in life have been ineffable and consistent encouragement from brother has been the pillar of my hold-up throughout.

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Chapter I General Introduction

1. Introduction

The theory of evolution brought us nearer to the question about the emergence of life and its origin. The biggest challenge in this endeavor was to unravel the chemical processes that made the synthesis of complex molecules containing information possible. In 1966, Norm Pace and Sol Spiegelman reported that ribonucleic acid (RNA) could serve as template for the synthesis of RNA. This report thus supported that RNA could be the instructive agent in a replication process and satisfy the operation of a self-replicating entity. Furthermore, this work provided the set-up for studying the genetics and evolution of a self-duplicating nucleic acid molecule. On the basis of this and other reports, Carl Woese, Francis Crick and Leslie Orgel suggested RNA to be the first informative molecule. The idea of the RNA world was the most exciting discovery based on the catalytic properties of RNA reported by the laboratories of Thomas Cech and Sidney Altmann. After the establishment of the RNA World hypothesis the experimental proof of concept on the evolution of a self-replicating informational system was challenging and required assemblage of all necessary tools to do so. In 1970, Howard Temin and David Baltimore discovered reverse transcriptase, an enzyme that can make deoxyribonucleic acid (DNA) from an RNA template. Furthermore, the purification of reverse transcriptase allowed for synthesis of DNA from RNA template was an essential tool for the development of *in vitro* evolution technology. Also, random synthesis of oligonucleotides and establishment of polymerase chain reaction (PCR) made it possible for specific amplification of DNA sequences. The above-mentioned discoveries paved the pathway for the invention of an ingeniously efficient method to amplify, mutate and select molecules with desirable properties[1–5].

In 1980, a study on the Human Immunodeficiency Virus (HIV) and adenovirus indicated that these viruses encode a number of small structured RNAs that bind to viral or cellular proteins with high affinity and specificity. In case of HIV, a short RNA ligand called trans-activation response (TAR) element promotes trans-activation and virus replication by binding with the viral trans-activator of transcription (Tat) protein. Similarly, adenovirus also has a short RNA

aptamer, virus-associated (VA)-RNA, that regulates translation[6–9]. In 1990, Gerald F. Joyce`s group at the Scripps Research Institute used the *in vitro* mutation, selection and amplification to isolate RNA with enzymatic functionality. Larry Gold`s group at the University of Colorado used a randomized eight-nucleotide sequence to identify sequences of T4 DNA polymerase *in vitro* and named the process Systematic Evolution of Ligands by Exponential enrichment (SELEX) (US Patent 5,270,163). J.W. Szostak and A.D. Ellington at the Massachusetts General Hospital (Boston) reported a library with 100 randomized nucleotides for the selection of target-organic dyes. The ultimate functional form of a protein is determined after the post-translational modifications. Henceforth, it is necessary to detect the proteins in order to fully understand the cellular function, detection of diseases or monitoring the progression of an existing disease state and the effect of a drug on an organism. Nucleic acid ligands evolved, termed as aptamers, originated from the Latin “aptus” meaning to fit and “meros” meaning part and SELEX, an *in vitro* selection technology for the separation of binding ligands from non-binding ligands was established[10–12].

Here, the basics of this unique technology and its development over the years have been reviewed. Moreover, the characteristic property of recognition molecule-aptamers showing resemblance to the antibodies have been discussed in detail. Also, a brief account of the aptamer products in clinical trials has been summarized. Other than this, the biosensing platform is an interesting aspect for the developing and already developed aptamers from point-of-care perspective. Thus, information about the much-studied aptamer sensors is also presented. These topics have been deliberately considered for understanding the limitations of the current technology alongside the great achievements.

2. *In vitro* selection

Aptamers

The concept of “magic bullet” was envisioned by German scientist Paul Ehrlich of medicine, where a treatment would target only the disease-causing agent with high specificity and affinity and leave the healthy tissue untouched[13]. The foundation of magic bullet and the accomplishment could be realized in monoclonal antibodies and eventually epitomized in aptamers as both are active in identifying the disease-causing agent with high affinity and

specificity, differentiating between the target. The following factors can be considered to contribute towards the binding affinity between aptamers and their targets: hydrogen bonding, structure compatibility, stacking of aromatic rings, electrostatic and hydrophobic interactions and van der Waals forces[14]. The widely accepted *in vitro* selection methodologies have been remarkable in discovery of binding bio-probes/aptameric reagents for a range of biomolecules such as simple ions[15], small molecules[11,16], peptides[17], single proteins[18,19], ,organelles[20], viruses[21] and even entire cells[18]. Nucleic acid aptamers are high-affinity nucleic acid ligands (20 to 60 nucleotides) selected through ssDNA or RNA binding a specific target molecule from a random pool *in vitro*. Along with DNA/RNA oligonucleotides, peptide molecules have also been acknowledged for their recognition characteristic. Peptide aptamers are combinatorial protein molecules with specific binding affinity to the target of interest isolated in intracellular condition. Basically, the peptide aptamer comprises of the short peptide region inserted within a scaffold protein. The short peptide region interacts with the target protein and the scaffold region enhance the binding affinity and specificity through restriction on the conformation of the binding peptide. Due to the scaffold integration, the smaller peptide entity is capable of strong interaction with the target molecule. Thus, the advanced properties of the peptide aptamers make them more relevant in terms of molecular interaction studies when compared to DNA/RNA aptamers. However, the stability of the DNA aptamers cannot be disregarded. The application of the peptide aptamers in biological study and therapeutics include *in vitro* detection of various proteins in a complex mixture to *in vivo* modulation on proteins and cellular functions[22].

Systematic evolution of ligands by exponential enrichment (SELEX)

Systematic evolution of ligands by exponential enrichment (SELEX) is a well-established and efficient technology for the generation of oligonucleotides with a high target affinity. The conventional SELEX methodology comprises of three main steps: binding, partitioning and amplification. Prior to the selection cycle, a library comprising of 10^{15} random unique sequences is synthesized. Each unique sequence contains random bases in the variable region generally of 20-50 nucleotides flanked by conserved primer binding sites on both ends which are used for PCR amplification by annealing primers. Firstly, for the binding step the library is incubated with the target molecules for indicated time. After incubation, the non-binding sequences are separated from the binding by partitioning methods. The number of rounds

necessary for selection can be determined by a variety of parameters such as the characteristic and composition of the target, library design, the conditions of selection, target-oligonucleotide ratio and the efficiency of the separation method. The stringency of SELEX process governs the affinity of the binding bio-probe to the target which is progressively increased over the successive rounds by changing the binding and washing conditions such as buffer composition, volume, time and or decreasing the target concentration in the final rounds of selection. Then the binding ligands are eluted and amplified by PCR/rtPCR for DNA/RNA oligonucleotide. This amplified pool of selected ligands is used for the next cycle of selection repeating the same steps as mentioned above. After several rounds of selection cycle, the enriched pool of sequences is further proceeded with cloning and sequencing and the selected aptamer candidates are then tested for their binding abilities (Figure1).

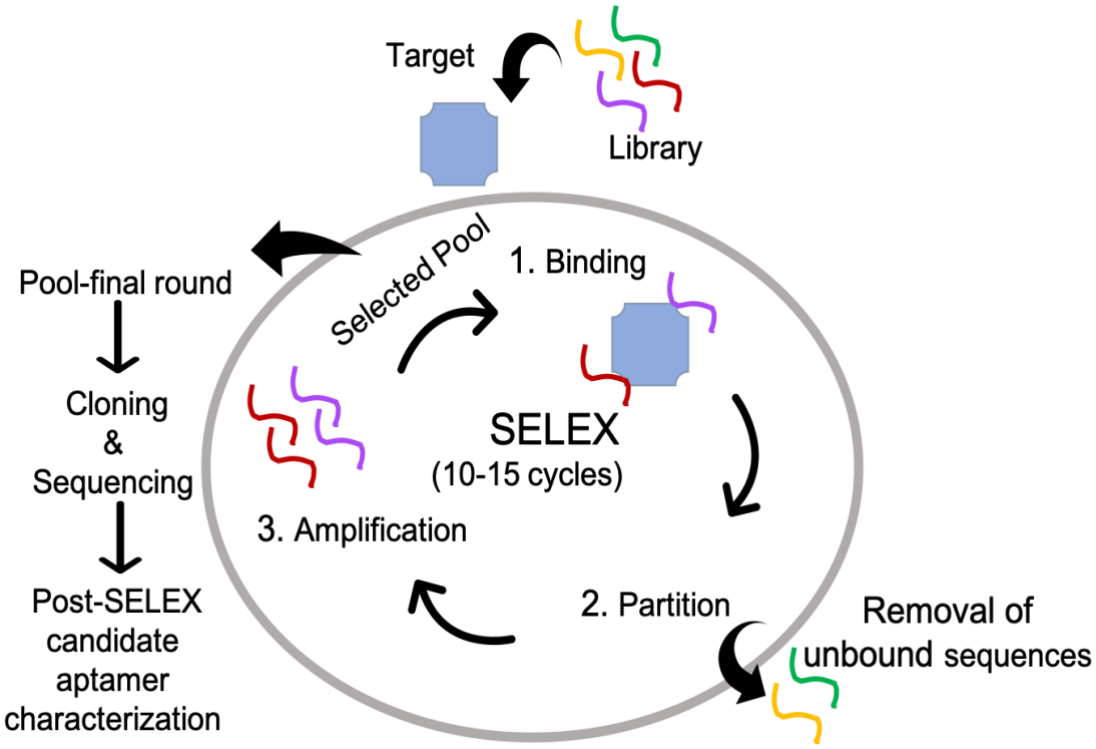


Figure1. Basic Process of Systematic Evolution of Ligands by Exponential enrichment (SELEX). The traditional method of SELEX comprises of three main steps:(i) binding, (ii)partitioning and (iii) amplification followed by cloning & sequencing. The obtain candidate are then evaluated for their binding parameters and evaluated for candidate determination post-SELEX.

Generally, the hit rate is low, however if a successful candidate is evolved then post-SELEX characterization of the aptamer is performed to enhance its functionality. Thus, the conventional approach offers a complex and tedious process and the identification of aptamer against relevant targets remains a challenge[23–25]. So, several modifications were developed, and new types of SELEX process have evolved in the last 29 years (Figure 2). Some of the well-established ones are, negative-SELEX[26], counter SELEX[27], capillary electrophoresis SELEX[28], microfluidic SELEX[29], cell SELEX[30], *in vivo* SELEX[31] and high-throughput sequencing SELEX[32] .

3. Development of *in vitro* selection technology

Vast interest in aptamers stimulated continuous development of SELEX, which underwent numerous modifications since its first application in 1990. Novel modifications made the selection process more efficient, cost-effective and significantly less time-consuming. Here a comprehensive and up-to-date review of recent advances in SELEX methods, pinpointing their advantages, main obstacles and limitations is presented.

Negative SELEX

In 1992, Ellington and Szostak introduced the method of eliminating false-positive results by performing negative SELEX. Generally, during the selection process some of the sequences might bind to the immobilization matrix enabling partitioning. So, after three selection cycles they incubated the library with purification support agarose as negative selection. After the removal of the non-specific binding sequences, the affinity of the resultant pool was enhanced by 10-times as compared to without negative SELEX[26].

Counter SELEX

In 1994, Jenison et al. introduced the counter SELEX[27] method in which an additional step of incubation with structurally similar targets is performed to enhance the specificity of aptamers. This method is beneficial to effectively discriminate against non-specific oligonucleotides. Counter SELEX is a kind of negative SELEX which uses similar configuration molecules for partitioning pressure. This method has been integrated with other modified SELEX technology such as cell-SELEX[33], immuno-affinity SELEX[34], quartz crystal microbalance (QCM) SELEX[35] to name a few to improve the selectivity of aptamers.

Capillary Electrophoresis SELEX

The conventional SELEX method requires 10-15 rounds of selection cycles to obtain aptamers thus making it more labor intensive and time consuming. Capillary Electrophoresis SELEX (CE-SELEX)[28,36], another modification of SELEX developed in 2004. In this method, the difference in the electrophoretic mobility of the target bound sequences from the unbound sequences was used for separation. This was a highly efficient method enabling selection of high-affinity aptamer candidates from 1-4 selection rounds. Furthermore, another CE-based technology called non-SELEX[37], selects an aptamer without amplification. This method accelerated the selection procedure and minimized the DNA amplification bias caused by repetitive steps of PCR. Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM), a highly efficient affinity-method was used to partition the oligonucleotides-target complex from the free oligonucleotides. The time for the selection process has been further reduced by this technology. However, limited volume of library can be injected into the capillary thus limiting the number of sequences.

Microfluidic SELEX

A microfluidic SELEX (M-SELEX) prototype[29] was developed in 2006 by Hybarger et al. combining traditional SELEX with microfluidic system. The prototype contains reagent-loaded micro-lines, a pressurized reagent reservoir manifold, a PCR thermocycler and actuatable valves for selection and sample routing. In 2009, Luo et al. described a more rapid, efficient and automatic aptamer selection system[38]. This system integrated the magnetic bead-based SELEX process with microfluidics technology and a continuous-flow magnetic activated chip-based separation device. Single round of selection was able to yield an enriched aptamer pool that could bind to recombinant botulinum neurotoxin type A with high affinity. However, the limitation of microfluidics such as aggregation of magnetic beads and microbubbles in the microchannel lead to distortion in flow streams might affect the aptamer purity and recovery process. However, these disadvantages were further improved by fabricating the microchannel with ferromagnetic materials by Soh et al. With this improved SELEX, they obtain aptamers with a Kd value of 25 nM in only three rounds of selection. Also, in 2009, Park et al. developed another novel microfluidic SELEX incorporating nanoporous sol-gel protein microarray[39] material which was able to hold a large number of target molecules enabling selection against multiple targets. Several target molecules with Kd in low nM range

have been generated using sol-gel SELEX[40,41]. However, the concerns of protein integrity and stability through multiple selection cycles in sol-gel SELEX still exist.

Cell SELEX

Based on the observation that selected aptamers *in vitro* fail to recognize and bind to the same protein at endogenous levels or cellular condition, cell SELEX was developed. The merit of this technology lies in the fact that it employs the whole live cells as target which increases the possibility of selected aptamer to be used directly for diagnostic and therapeutic applications. Also, the molecular targets are in their native conformation thus the obtained aptamers represent the eventual results. There is no requirement for protein purification or prior-knowledge of the molecular targets on cell surface and this process can pave pathway for discovery of new biomarkers and unknown surface proteins. The first report of cell-SELEX was in 2003 by Daniels et al. for successfully obtaining a DNA aptamer against tenascin-C using a glioblastoma-derived cell line, U251[30]. Ohuchi et al. designed a novel SELEX method TECS SELEX[42], in which a cell-surface displaying recombinant protein was directly used as selection target thus combating the time consuming and difficult process of purifying proteins. Other methods based on cell-SELEX such as FACS-SELEX[43], 3D cell SELEX[44] and cell-internalization SELEX[45,46] have also been developed.

In vivo SELEX

For the functionality of aptamers *in vivo* the aptamers selected *in vitro* might not be purposeful thus leading to development of *in vivo*-based SELEX method for generating tissue-penetrating aptamers directly within animal models of the target disease. Mi et al. firstly tried to select aptamers inside a tumor of a living organism in 2010[31]. A library of 2`-fluoropyrimidine-modified RNA aptamers was injected into the tail vein of intrahepatic tumor-bearing mice. Then the aptamers were extracted from the liver tumors, amplified and re-injected into other similar tumor-bearing mice. With this method they could successfully select aptamers against p68 and RNA helicase with Kd values in nM levels. Another example is of Cheng et al. identifying aptamers that could bind to brain capillary endothelia and penetrate into the parenchyma demonstrating feasible generation of aptamers using live-animal model as selection targets[47].

One round SELEX

Several reports showing selection of high affinity aptamers in Nano-molar affinity range with one round selection have been reported. The MonoLEX method is a straightforward procedure to isolate high-affinity DNA aptamers for Vaccinia virus. This comprises of single affinity chromatography step, followed by segmentation and signal final PCR amplification step of bound aptamers. This process highlights the reduction of competition between aptamers of different affinities during PCR step, advantage for the single round selection[48]. In another method DNase-based digestion was used for selection of proteins blotted on membrane. Unbound and weakly bound sequences were efficiently removed[49].

Artificial/non-natural and modified nucleotide SELEX

To increase the nucleotide repertoire with only four natural nucleotides for selection of bunding ligands, artificial and non-natural nucleotides[50] are being employed for selection process. Also, artificially expanded genetic information systems (AEGISs)[51] have been innovated. However, such systems can be used for few targets and limited due to activity of polymerase and tagging process essential to perform multiple selection rounds.

High-Throughput Sequencing SELEX

The identification of the candidate aptamer in the pool after the final selection round is mainly done by Sanger sequencing. However, in most cases the aptamers in the final pool are not the ones with high affinity and specificity. Thus, it becomes difficult to analyze which is the best aptamer. Recently, a new technology, high-throughput sequencing (HTS) was introduced which allows for sequencing the library across all the selection rounds thus remarkable for analysis of the enriched sequences at early stage. This method is time efficient and assists in avoiding PCR bias caused by over selection. Also, large scale analysis of sequence datasets by robust bioinformatics tools further facilitates comprehensive characterization of aptamers including binding affinity and /or specificity, structure prediction, abundance quantification and aptamer-target interactions[52]. In 2010, Cho M et al. showed the first application of high-throughput sequencing[32]. Several aptamers against different targets have been identified using HTS-SELEX[53–55].

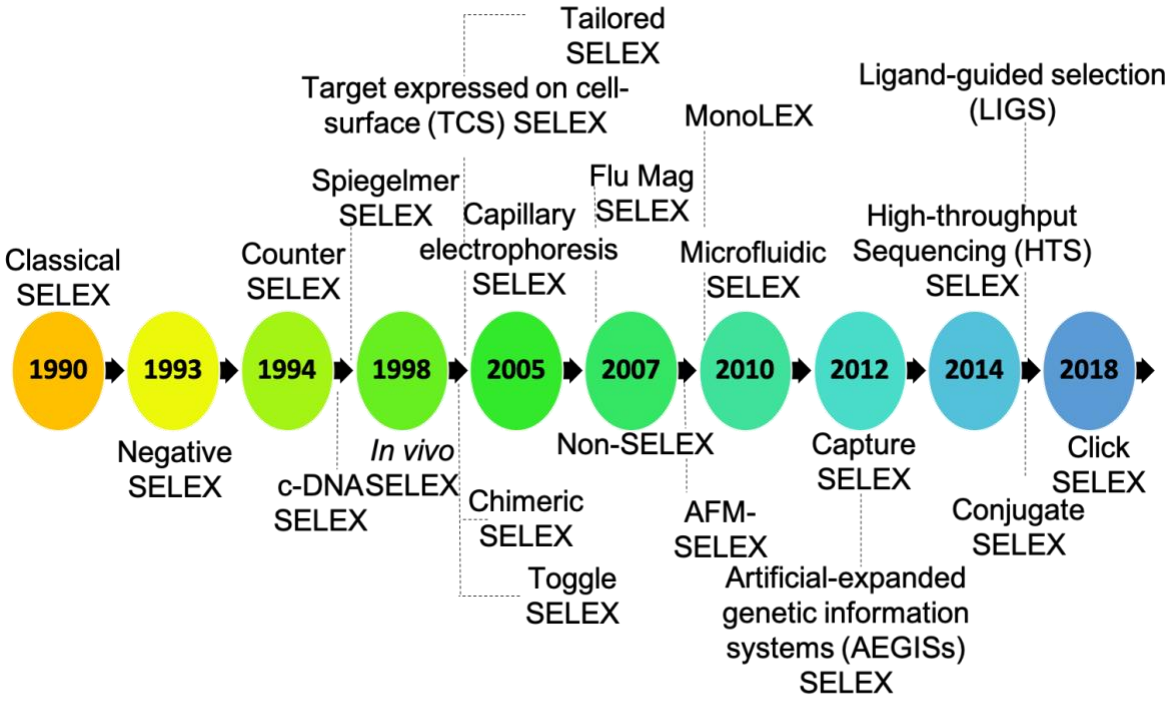


Figure 2. The timeline for the development of SELEX technology from beginning. This flow-chart demonstrates the modification of SELEX developed with the progressing time to meet the challenges of conventional technique. An attempt to list all the major discovery in the course of progression has been made.

4. Application of aptamers

After three decades of antibodies mediated sensing technology, aptamers are being well recognized at the forefront of the detection technology due to their advantageous characteristic traits such as high affinity and specificity. Aptamers have been yielded with affinity in the μM range for small molecules such as dopamine (2.8mM), ATP (6mM) and nM-pM range against proteins such as VEGF, KGF[56,57]. In general, high affinity is considerable as high-specificity for a variety of targets, thus reporting the aptamers that can discriminate between targets using subtle structural differences such as presence of hydroxyl group or a methyl group, enzymes with similar catalytic function a-, g- thrombin and also enantiomers. In the case of caffeine and theophylline which differ only in a methyl group, aptamers demonstrated a high molecular discrimination as compared with antibodies[27,58,59]. Aptamers can be generated that bind essentially any target with high-affinity, thus similar in their

function to monoclonal antibodies and this property enhances the number of clinical indications that are potentially detectable by these nucleic acid-peptide compounds. Henceforth, aptamers can be considered as nucleotide-analogues of antibodies. And in the best of our interest, these engineered biomolecules have several properties that enhance their applications, better than the competitor antibody molecule. The process of identification of the antibody is a long and complex one and dependent on the host animal. Moreover, antibody generation process is furthermore complicated in the case of toxic targets, that can be harmful for the host or low molecular weight compounds which trigger a minimal immunogenic response. Also, antibody show batch to batch variations which have been reduced by the monoclonal antibody preparations, however the purification and processing process is a lot more tedious. On the other hand, aptamer-generation is significantly easier and cost-effective as compared with the production of antibodies. They are selected by the *in vitro* selection strategy and high-throughput screening systems independent of a host. They are non-immunogenic and non-toxic. After post-SELEX characterization of selected aptamer, they can be generated accurately and reproducible by automated chemical synthesis. Moreover, the aptamer selection strategies can be defined and monitored for the selection of candidate aptamer with desirable traits[60–62]. Aptamers can be renatured easily and do not lose their functionality in varied temperature and physiological conditions that would otherwise denature the antibodies permanently. Antibodies are large and complex molecules sensitive to non-physiological pH and temperature, thus limiting their potential to be reusable. On the other side, aptameric reagents can undergo reversible denaturation and renaturation, not affecting their structure. Furthermore, the kinetic parameters can be changed on demand in an aptamer-based interaction and they can be easily transported at ambient temperature without degradation and stored in cold and subjected to numerous freeze-thaw cycles. These smart biomolecules have been widely applied in analytical, bioanalytical, imaging, diagnostic and therapeutic fields[63].

Developed aptamers in clinical trials

In the present situation, the aptamer science is amplifying/becoming larger with the rate of minimum one new aptamer research being reported each day. For the accumulation of such substantial data a separate aptamer database has been created for ease of theoretical biologists[64]. Several successfully emerging aptamers have found their position in the stage of

clinical trials for specific conditions, thus demonstrating the factual importance of the aptamers in clinics. Based on their properties of being able to block the receptors and inhibit the protein activity with high affinity and specificity they have been well-applied in the discovery of therapeutic drugs. Macugen (OSI Pharmaceuticals, Melville, NY) is a good example of a successful aptamer based therapeutic for the treatment of age-related macular degeneration. This aptamer inhibits the anti-vascular endothelial growth factor, that participates in the growth of abnormal blood vessels in the eyes that cause vision loss. Macugen has been approved by FDA for patients with neo-vascular age-related macular degeneration[65]. There are several other reports of aptamers undergoing clinical trials (Table 1). Protein tyrosine kinase-7 (PTK7) is reported to play an important role in motility and invasity of cancer cells and it overexpresses in different human cancers. Sgc8, a 41 oligonucleotides ssDNA aptamer selected by cell-SELEX is a specific ligand of PTK7. In this study, Sgc8 was linked to a bi-functional group NOTA for 68Ga chelation for the detection of colorectal cancer (CRC), third common cancer and fourth leading cause of death worldwide. PTK7 is overexpressed in CRC and correlated with tumor differentiation, lymph node metastasis, distant metastasis stage of CRC patients. The clinical application of 68Ga labeled ssDNA aptamers Sgc8 will be studied in healthy volunteers and colorectal patients (NCT03385148). EYE001, an anti-VEGF Pegylated aptamer was used for a pilot study of intravitreal injection for advanced ocular disease of Von Hippel-Lindau (VHL) to treat retinal tumors in patients with Von Hippel-Lindau syndrome (NCT00056199). EYE001 decreases production of VEGF, a growth factor important for formation of new blood vessels which is elevated in VHL. Findings from studies of retinal diseases have suggested that EYE001 can reduce retinal thickening and improve vision. Archemix Corp., ARC-1779 is an optimized, second generation, PEGylated aptamer that exerts a novel antithrombotic action through targeting the A1 domain of activated von Willebrand factor (vWF). ARC-1779 has potential therapeutic benefit in acute coronary syndromes and von Willebrand`s disease as well as in vWF-related platelet disorders such as thrombotic thrombocytopenic purpura (TTP) (NCT00632242). The actions of ARC-1779 can be readily reversed by binding to complementary sequence of oligonucleotides thus offering potential therapeutic benefit in surgery[66]. In a second clinical study of NOX-A12, the safety, tolerability and pharmacokinetics and effect of mobilization of hematopoietic stem cells of NOX-A12 alone and in combination with Filgrastim was done (NCT01194934). In another study, the REG-1 aptamer-RNA target to

Factor IXa was evaluated (NCT00113997). The anticoagulation system REG-1 was designed to improve control of blood thinning. The REG1 system is designed such that one part of the system stops the activity of factor IX (protein that helps blood clot)[67] while the other part of the system (the antidote) inactivates the drug and stops the thinning process. ARC1905, an anti-C5 aptamer was tested in combination therapy with Lucentis®0.5 mg/Eye in subjects with subfoveal choroidal neovascularization secondary to age-related macular degeneration (AMD) (NCT00709527).

Although aptamers have several merits in therapeutics, short half-lives, nuclease degradation and rapid renal clearance have limited their efficiency *in vivo*. However, to remove such barriers various modifications and conjugations of aptamers have been adopted. Most aptamers in clinical studies have been modified in general at (i) ends of nucleic acid chain, (ii) sugar ring or nucleoside and (iii) phosphodiester linkage[68,69]. The aptamers are easily excreted through renal filtration because of their small size. So, the main strategy to avoid this is attachment of bulk moieties such as cholesterol[70], polyethylene glycol (PEG)[71], proteins[72], liposomes[73], organic and inorganic materials[74]. Aptamer can also be used as targeted drug delivery system, due to specific binding to a target molecule or an intended site. Based on the delivery agents by the aptamers, the aptamer targeted drug delivery systems have been classified in three major categories: aptamer-small molecule, aptamer-RNA and aptamer-nanomaterial conjugated systems.

Table1. Developed aptamers advantageous for in vivo diagnostic applications in clinical trials[1]

Aptamer/Drug	Form	Target	Condition/Disease	Phase
68Ga-Sgc8	DNA	Protein tyrosine kinase-7 (PTK-7)	Colorectal Cancer	Early Phase 1
EYE001	RNA	VEGF (Decreases production)	Von Hippel-Lindau (VHL) Disease	Phase 1
ARC1779	DNA	A1 domain of activated vWF (von Willebrand factor)	Purpura, Thrombotic Thrombocytopenia, Von Willebrand Disease Type-2b	Phase 2
NOX-A12	RNA	CXCL 12 Mobilization of HSC (hematopoietic stem cells)	Hematopoietic Stem Cell Transplantation	Phase 1
REG-1	RNA	Coagulation Factor IXa	Anticoagulant for conditions such as heart attack and another coronary artery disease	Phase1
E10030	DNA	Platelet-derived growth factor (PDGF) (Anti-PDGF pegylated aptamer)	Age-related macular degeneration	Phase 1
ARC1905	DNA	Complement factor C5 (Anti-C5 aptamer)	Age-related macular degeneration	Phase 1

[1] This data has been collected from the site of clinicaltrials.gov

Aptasensors in point-of-care testing

In addition to the other applications, substantial progress has been made in the development aptamer-based sensors for the diagnostic/detection purposes (Table2). Aptasensors are biosensors based on aptamers as recognition element. The critical characteristic for aptamer biosensors is their specificity independent of physical parameters such as pH, temperature. Aptasensors were first reported in 1966, in an optical sensor system, consisting of human-neutrophil elastase coated beads that interact with fluorescent-tagged aptamers. Further, an aptasensor was developed in which a radiolabeled aptamer was used for the detection of protein kinase C isozymes and another in which an enzyme-linked sandwich assay used a SELEX-derived fluorescently labeled oligonucleotide[75–77].

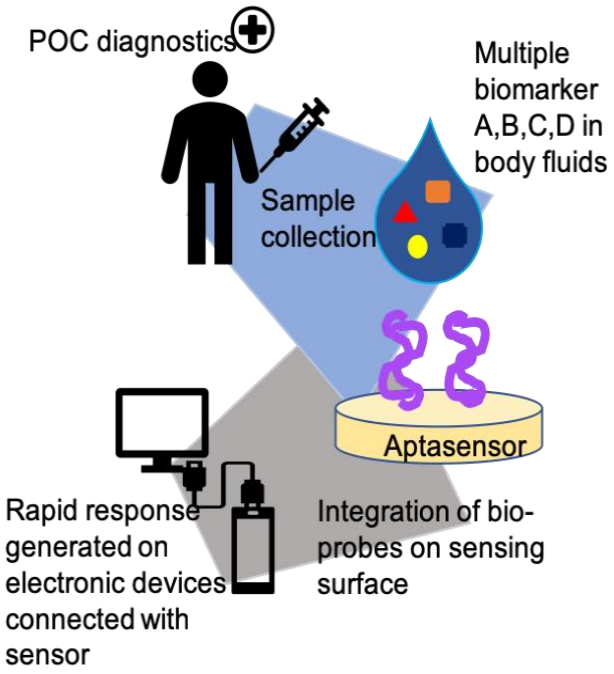


Figure 3. Application of aptamer-based sensors in point-of-care testing. This figure is demonstrating the application of the aptamer developed by SELEX technology for detection of specific biomarker in clinical sample and allow rapid and reliable point-of-care diagnostics by integration of aptamer as sensing element.

A variety of methodologies such as electrochemical biosensor, optical and mass-sensitive biosensor are employed for the construction of aptasensors. Novel microfabrication

technologies based on electrochemical analysis using several techniques such as electrochemical impedance spectroscopy (EIS), potentiometry with ion-selective electrodes (ISEs), electrogenerated chemiluminescence (ECL), cyclic voltammetry (CV) and differential pulse voltammetry (DPV) are attractive biosensing platforms with high sensitivity, compatibility, miniaturization and low-cost[78–83]. The first report of the aptasensors based on electrochemical transduction phenomenon was reported in 2004 by Ikebukuro et al. Since then the affinity-based biosensing platform has been continuously evolving in the field of clinical diagnostics, environmental monitoring and point-of-care testing[84–86].

There are hundreds of publications in the field of aptasensors in the past decade, with different transduction platforms. Amidst the discovered aptasensors, electrochemistry is the most promising field providing innovations with high specificity, reasonably low prices and possibility of miniaturization. This is mainly because of the low-cost production of micro-electronic circuits and their convenient interface with normal electronic-readout and processing. The group of Plaxco et al. are pioneers in development of label free biosensors, effective for the recognition of thrombin by electrochemical detection approach. In such biosensors, the DNA aptamer is immobilized on the electrode surface from one end and linked to a redox label on the other end. The redox probe is either activated or deactivated by the formation of the aptamer-target molecule complex, thus measuring the changes in the redox activity by highly sensitive electrochemical technique, AC voltammetry. In 2006, Xiao et al, developed an electrochemical aptamer-based sensor comprising a redox-tagged DNA aptamer directed against blood clotting enzyme thrombin. The thrombin binding reduces the current from the redox tag, readily signaling the presence of the target. In another work, by Cash et al 2009, the double stranded DNA was used as a support scaffold for the small molecule receptor, sensors for detection of protein-small-molecule interactions were fabricated for detection of low nM concentration of antibodies against the drug digoxigenin. Quartz crystal microbalance (QCM) and surface plasmon resonance (SPR) are two widely described techniques for transduction and detection of non-labeled aptamers. A microfabricated cantilever-based sensor functionalized with aptamers as receptors of Taq DNA polymerase uses the label-free protein detection strategy. Also, atomic force microscopy (AFM) has been used to measure the specific interaction between a protein immunoglobulin

E (IgE) and an aptamer. In addition, a surface acoustic wave biosensor array couples' aptamers to detect thrombin and HIV-1 Rev peptide[87–89].

Table2. Recent reported studies of aptamer-based sensors for clinical point of care testing application

Biomarker/Condition	Aptamer	Detection	Transduction	LOD/Range	Reference
Prostate Specific Antigen (PSA)/Prostate Cancer	DNA	GQDs-AuNRs	CV, DPV, EIS	0.14ngmL^{-1}	90
Osteopontin/Breast Cancer	RNA	μ-AU electrode	CV	8nM	91
CCRF-CEM cell/ Leukemia	Sgc8	GO-apt-FAM	FRET	10 cellsmL^{-1}/10^{2}to 1*10^{7} cellsmL^{-1}	92
C-reactive Protein/ Cardiac Disease	RNA	SiMSs-Au NPs	SWV	0.0017ngmL^{-1}/0.005ngmL^{-1} to 125ngmL^{-1}	93
Vascular endothelial growth factor-165 (VEGF$_{165}$)/ Cancer Angiogenesis	-	Nanoplasmonic	Optical	25pgmL^{-1}- 25ugmL^{-1} (comparable to ELISA)	94
Haemagglutinin protein (HA)/ H1N1 Flu	aHP(DNA)	GO/KF-polymerase strand displacement	Fluorescence	(HA)2.5μgml^{-1} / (H1N1 virus)1*10^{2} TCID50	95
AIV H5N1/ Avian Flu	DNA	Au-stav-bio-apt	SPR	0.128 to 1.28 HAU	96
Human Immunodeficiency Virus type 1 Trans-activator transcription protein (HIV-1 Tat)/ HIV	RNA	Diamond-FET	Potentiometric	-	976
Hepatitis C Virus (HCV)/ Hepatitis	-	GCE-GQDs	EIS, CV, DPV	3.3 pgml^{-1}	98
Plasmodium falciparum glutamate dehydrogenase (P*f*GDH)/ Malaria	DNA	aptaFET/ IDμE (Intedigitated gold micro electrodes)	Potentiometric	48.6pM/100fM to 10nM	99
Murine Noriovirus (MNV)/ Viral gastroenteritis	AG3(DNA)	GNPs-SPCE	SWV	180 virus particles	100
Glycated Human Serum Albumin (GHSA)/ Diabetes Mellitus	DNA	GO-G8apt	Fluorescence	50 μgmL^{-1}	101

5. Impediment of aptamer development and subsequent outlook

Conclusion

From the study of literature about SELEX and aptamers, it can be assumed without any doubt that remarkable achievements have been made since the first report in 1990. The recent developments of making the selection process more visible and clearer at each step with high-throughput sequencing and binding analysis with different techniques such as Isothermal Titration Calorimetry (ITC), Microscale Thermophoresis (MST), Surface Plasmon Resonance (SPR) and Flow-cytometry mark the development of this technology leading to successful isolation of aptamer candidates for real-time applications. However, despite the great advances coming to the forefront of aptamer selection and application, few aptamers have successfully commercialized. On observation, following are some of the listed factors responsible for the setbacks to be considered and evaluated (Table 3).

Table 3. List of several challenges in the SELEX technology in present scenario

S.No.	Challenges
i.	The traditional SELEX process is still complex, time consuming and high cost
ii.	The successful rates are low in general
iii.	Repeated amplification in each cycle might induce PCR-bias
iv.	Most selected aptamer have high-affinity and low-specificity
v.	Most aptamers are obtained *in vitro* thus shrinking their efficacy *in vivo*
vi.	Molecular size of aptamers reduces their *in vivo* applications thus demanding post-SELEX optimization
vii.	Restricted amplification of modified and/or unnatural nucleic acid with polymerase
viii.	The dominating antibody-market and lack of dependency on aptamer due to unfilled gaps restricts the overall leap towards aptamer commercialization

Research Outline

In chapter I, the development of SELEX is reviewed, and the key aspects of this technology are described. Also, the barriers for the evolution of aptamers in the present scenario have been assessed.

In chapter II, an alternative strategy for the selection of aptamer, termed as Systematic Evolution of Ligands by Competitive enrichment (SELCO) has been demonstrated. The novel characteristic of competition-induced selection in presence of competitor target (similar configuration) has been considered based on theoretical explanation. The supportive experimental results in the preliminary stage have been reported here.

In chapter III, the successful identification and evaluation of aptamers selected via novel concept of SELCO has been described. The factual experiments for identification of aptamer and evaluation of structure and kinetics are reported here.

In chapter IV, the development of electrochemical sensor for evaluation of specificity and point-of-care application has been demonstrated. The results of successful model aptamer selected via SELCO integrated with original developed DEPSOR-mode electrochemical sensor for quantitative analysis have been reported here.

In chapter V, a summary of challenges, measures and inferences developed throughout the course of this research have been described. Also, an account of future perspective for this novel technology has been added.

Chapter II Development of competitive selection, SELCO (Systematic Evolution of Ligands by Competitive enrichment) for the selective and rapid enrichment of DNA aptamers

1. Introduction

An *in vitro* selection termed SELEX (Systematic Evolution of Ligands by EXponential enrichment) has allowed researchers to identify a diversity of DNA/RNA aptamer molecules. SELEX is operated using an iterative cycle of three fundamental steps, namely binding, partitioning, and amplification, and it can gradually enrich target-binding DNA/RNA molecules over the selection cycle[12,63,102]. Although the SELEX protocol has long been performed with success[14,103], the difficulty involved in selecting aptamers with high specificity remains[104]. The current approach to this problem uses "negative selection," which is universally applied to select aptamers that bind to a molecule of interest from a pool of non-bound molecules to a particular target of no interest (thus, they are negatively selected). This approach is widely applied, and in the case of SELEX, for example, there are reports that negative selection had the greatest positive results in selecting for cell-specific aptamers[105]. Although this approach is useful, in principle, it requires multiple rounds of negative and positive selections. The SELEX process essentially requires many rounds of selection using PCR, leading to the amplification of undesired biases[106–108]. Unfortunately, the final success ratio of SELEX-based experiments has not been high[109,110] although some cases were clearly successful[111,112]. Therefore, SELEX-based technology requires some effective improvements.

Here, we propose a novel approach for obtaining selective aptamers without PCR amplification procedures, namely 'SELCO' (Systemic Evolution of Ligands by Competitive enrichment), in which *in vitro* selection is performed using a solution system containing all the positive and negative targets. The rapid, precise, and selective detection of viruses is absolutely required to prevent breakouts/pandemics. This is especially true of the highly infectious influenza virus. In this chapter, we showed the plausibility of using SELCO on close targets of influenza virus subtypes (H1N1 and H3N2). On the whole, a powerful approach for rapid selection of aptamer with high sensitivity is presented here, and it addresses several theoretical considerations.

2. Materials and Methods

Chemicals and Reagents

The recombinant protein Influenza A H1N1 (A/California/04/2009) Hemagglutinin/HA Protein (His Tag) and Influenza A H3N2 (A/Aichi/2/1968) Hemagglutinin/ HA Protein (His Tag) were purchased from Sino Biological.The Ni-NTA magnetic beads were purchased from QIAGEN (Hilden, Germany). . Ex Taq HS DNA polymerase was purchased from Takara Bio (Kusatsu, Shiga, Japan). The respective oligo-sequences were ordered from Eurofins (Tokyo, Japan).
The streptavidin magnetic beads (1 µm) were purchased from New England Biolabs (Ipswich, MA, USA).

Immobilization of target molecules on Ni-NTA beads

To perform SELCO, we used the closely related subtypes of the influenza A virus H1N1 and H3N2. The targets H1N1 (abbreviated as T_{H1N1}) and H3N2 (abbreviated as T_{H3N2}) were immobilized onto Ni-NTA magnetic beads (20–70 µm) and Ni-NTA agarose resin beads (45–165 µm), respectively, according to the protocol for immobilizing the protein target stated by the manufacturer.

Library Design and Primers

The DNA library used for the selection was made up of a random 30-nucleotide region flanked by a 20-nucleotide primer region on both sides, specifically, 5'-AGCAGCACAGAGGTCAGATG(N30)CCTATGCGTGCTACCGTGAA-3'. For PCR amplification, the forward primer 5'-AGCAGCACAGAGGTCAGATG-3' and the biotinylated reverse primer 5'-TTCACGGTAGCAGCGATAGG-3' were used.

In vitro selection process by exponential enrichment

The plus strand ssDNA pool was heated to 90°C for 5 min and immediately cooled to 4°C and placed for 15 min, followed by incubation at 25°C for 15 min. Following this step, the target T_{H1N1} was immobilized on the Ni-NTA beads were incubated with 100 pmol of the ssDNA initial pool in the presence of the binding buffer (PBS buffer (pH 7.4), 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$) for 60 min. The supernatant was then removed by washing four times with washing buffer (PBST buffer (pH 7.4 with 0.05% Tween20), 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$). The selected aptamer DNA pools, which are bounded on beads, were recovered by heat treatment (90°C for 5 min followed by immediate removal of the supernatant). Further the selected pool was amplified with Ex Taq HS DNA Polymerase. PCR annealing temperature were studied for a varied range from 50ºC to 65ºC. The amplification

reaction was optimized at 98ºC for 2 minutes followed by 20 cycles of 98ºC for 10 seconds, 59ºC for 5 seconds and 72ºC for 10 seconds finally 72ºC for 4 minutes after the last cycle. BIOTIN-labelled ds-DNA pool candidates after each round of amplification cycle were bound to the streptavidin magnetic beads (1 µm) by incubation for 15min at room temperature following which the ss-DNA was separated by heating at 90 ºC for 5 min. The separated ss-DNA pool was collected with the help of magnet stand and used for the next round of selection. A total of 4 rounds of selection were performed with gradual decreasing incubation time and increasing wash-steps.

Table 4. Selection conditions for each round SELEX for subtype-H1N1 of Influenza A virus

Selection Round	**Incubation-time**	**Wash-steps**
1	60 minute	4 wash
2	45 minute	4 wash
3	30 minute	5 wash
4	15 minute	6 wash

In vitro selection process by competitive enrichment

The plus strand ssDNA pool was heated to 90°C for 5 min and immediately cooled to 4°C and placed for 15 min, followed by incubation at 25°C for 15 min. Following this step, the targets T_{H1N1} and T_{H3N2} that were immobilized on the Ni-NTA beads were incubated with 100 pmol of the ssDNA initial pool in the presence of the binding buffer (PBS buffer (pH 7.4), 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$) for 60 min. The supernatant was then removed by washing three times with washing buffer (PBST buffer (pH 7.4 with 0.05% Tween20), 100 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$). In each wash, the sample solution was briefly centrifuged at 1000 *g* for 10s and the supernatant was removed carefully. The same procedure was repeated by 4 rounds with a successive addition of 200 pmol, 400 pmol, and 800 pmol of the ssDNA pool, changing the incubation time and washing frequency 30 min (3 times washing), 15 min (2 times washing), and 7.5 min (1 time washing), respectively. Finally, both the targets immobilized on the magnetic or nonmagnetic beads were separated by magnetic force using a magnet stand or centrifugation force (1000 *g* for 10 s), respectively,

followed by the removal of the supernatant. The selected aptamer DNA pools, which are bounded on beads, were recovered by heat treatment (90°C for 5 min followed by immediate removal of the supernatant). The selected aptamer DNA pools for T_{H1N1} and T_{H3N2} were then briefly incubated with the crude Ni-NTA beads for 15–20 min in order to remove any nonspecific candidates, if exists. The specific DNA pools selected against T_{H1N1} and T_{H3N2} were then briefly incubated with the different target solution, T_{H3N2} or T_{H1N1}, to remove false positives. The specific pools for each target selected by SELCO were amplified by PCR (initial incubation at 98°C for 2 min, followed by 20 cycles of 98°C for 10 s, 59°C for 5 s, and 72°C for 10 s, and finally, 72°C for 4 min). Gel electrophoresis was used to monitor the successful amplifications using 8% polyacrylamide gel with 8 M urea at a temperature of 60°C.

SPR Measurements

The SPR measurement was performed using a BIACORE X100 instrument. A sensor Chip-NTA and NTA reagent kit (GE Healthcare, Uppsala, Sweden) were used for the immobilization of the His-tag protein target for the interaction studies according to the manufacturer's instructions. The running buffer HBS-P was used for all the experiments and 0.35 M EDTA was used for regeneration. The single-cycle mode was performed to compare the pool for T_{H1N1} selected by conventional method and SELCO. For this, 4 independent experiments were performed for the immobilization of ligand H1N1 (0.01 mg/mL) in the running buffer onto the sensor surface at a level of 2500–3000 RU, with a contact time of 60 s and stabilization period of 60 s. The different analytes used for comparison were the random library, pool for H1N1 selected by conventional method, pool for H1N1 selected by SELCO, and pool for H3N2 selected by SELCO. The selected ssDNA pool solutions of the following concentrations: 37, 7.4, 1.48, 0.296, and 0.0592 µg/mL were prepared in the running buffer and sequentially injected, starting with lowest concentration, at a flow rate of 30 µL/min for 60 s, followed by 60 s of dissociation. The kinetics of the association and dissociation were studied and compared.

Electrochemical Measurements

A disposable three-electrode screen-printed chip obtained from Biodevice Technology, Co. (Ishikawa, Japan), was used for this experiment. The disposable electrochemical printed (DEP) chip works on the principle of three-electrode system for electrochemical analysis, with a carbon-based working electrode (3 mm in diameter), a counter electrode, and an Ag/AgCl reference electrode. Two µL of the recombinant proteins H1N1 and H3N2 at a concentration of 0.25 µg/µL were dropped onto the working electrode of the DEP chip, which was then incubated for one hour at 4°C. This incubation allowed for the passive adsorption of the target protein onto the working electrode surface. After the incubation, excess target protein was rinsed three times with 100 mM PBS, and the chip was dried by gentle-blowing air. To suppress nonspecific adsorption, 3.5 µL of blocking buffer (100 mM PBS containing 1% BSA) was added to the chip; it was then incubated overnight at 4°C. For the electrochemical analysis, the chip was further rinsed three times with 100 mM PBS buffer and dried before it could be used for the assay. A 2-µL sample made up of Au nanoparticles conjugated to the selected DNA aptamer pool candidates was dropped onto the target-modified DEP chip surface; the chip was then incubated for 15 min at room temperature. It was then rinsed three times with 100 mM PBS buffer and connected to an electrochemical analyzer system (Model 650 A, CH Instruments, Inc., Austin, USA). Thirty µL of 0.1 M HCl was dispensed onto the DEP chip to electro-oxidate the AuNPs at a constant potential of +1.4 V for 40 s, immediately followed by DPV detection from +0.6 V to 0 V, with a step potential of 4 mV, a pulse amplitude of 50 mV, and a pulse period of 0.2 s. The selected pools by SELCO for T_{H1N1} and T_{H3N2} were tested and for specificity validation, the selected pool for T_{H1N1} was reacted with the T_{H3N2}-modified DEP chip and vice-versa. All the experiments were repeated three times to confirm the consistency of the analysis.

3. Results and Discussion

In vitro competitive selection

As shown in Figure 4, the pool of ligands (aptamer candidates) consists of various molecules that can be named L^{S}, L^{S1}, L^{S2}, $L^{S1/S2}$, L^{C}, $L^{S/C}$, and L^{X} depending on their binding nature in relation to the target molecules T_{α} and T_{β} (see details in the legend to Figure 4). Clearly, there is a difference in their behaviors under conventional SELEX and SELCO, which holds two or more target molecules. Those targets compete with one another for common ligands (especially, L^{S}, $L^{S1/S2}$ and $L^{S/C}$) that can bind both targets T_{α} and T_{β} during SELCO but exclusively T_{α} in conventional SELEX. This characteristic is the origin of the name "SELCO". For this reason, the ligands that bind to the S_1 site (i.e., a T_{α}-specific site) are decreased to half except L^{S1} (which binds exclusively to S_1 site), resulting in enriched L^{S1}. Clearly, this effect cannot be expected from conventional SELEX. Therefore, in the equilibrium state of the interaction between the targets and the pool of ligands, we can expect a more L^{S1}-enriched (in other words, T_{α}-specific ligand-enriched) result from SELCO than SELEX. Under our experimental conditions (see the protocol in Methods and Figure 5), the near-saturation of binding sites with ligands is expected to be attained (an 8-fold excess of ligands against a target molecule at the final stage). The selection products (ligands) obtained in this way were processed for a negative selection (the selected ligands were treated with a mixture of all the possible targets except the genuine one and then the nonbinding ligands were collected), although this process is theoretically omittable. Note that SELCO procedure does not depend on the PCR amplification, which is a prominent difference from conventional SELEX (see Figure 5) and as also discerned earlier by protocol of non-SELEX[113]. This property simplifies the whole procedure and saves experimental cost when selecting DNA aptamers. Incidentally, several studies have supported the idea that the presence of competitor molecules can enhance the specificity of the selected candidate[114–116] though none has highlighted on the competitive effect pointed out in the work.

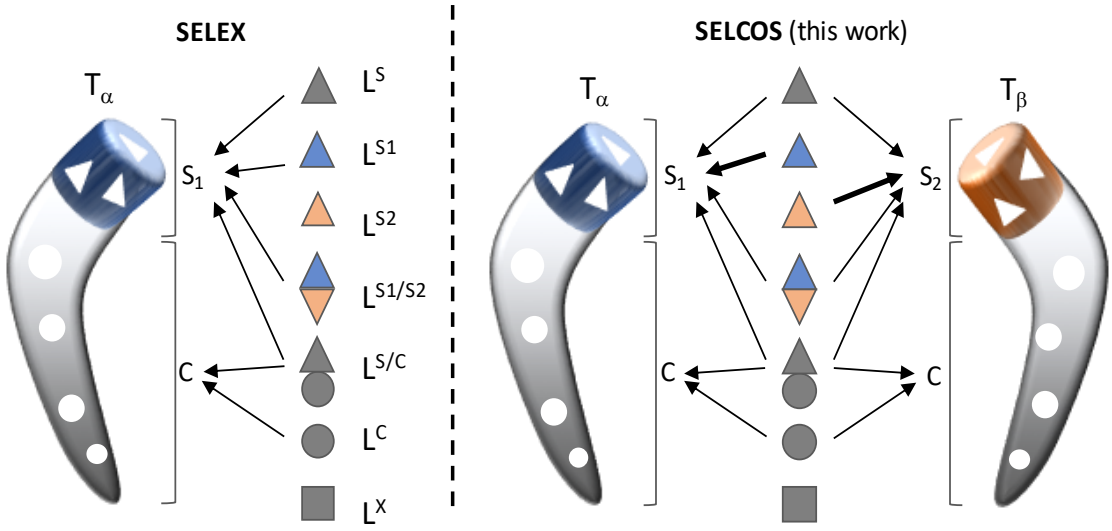
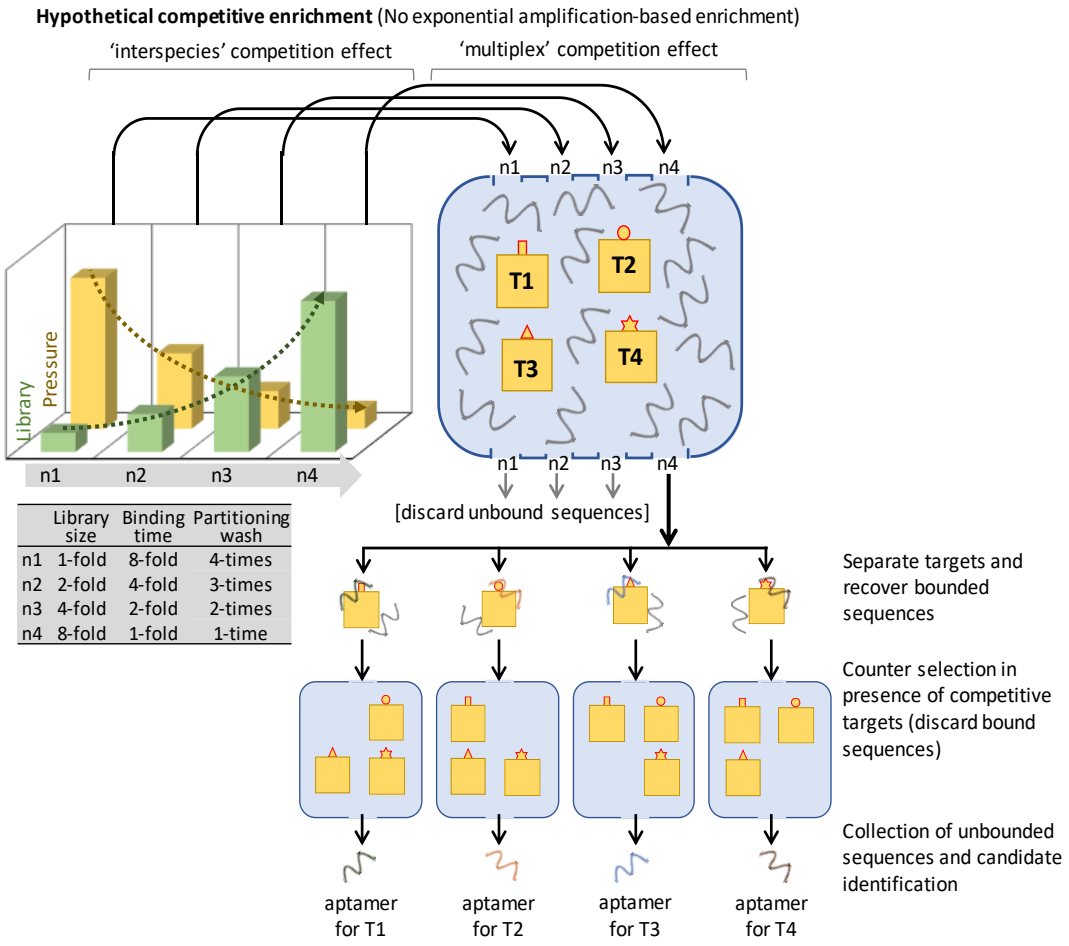


Figure 4. Schematic drawing of SELCOS (competitive non-SELEX). Comparison of (conventional) SELEX and SELCO in the ligand binding mode to the target protein. A pool of ligands is classified into 7 types in their binding mode to two different targets (T_α and T_β), which are composed of the common site (C) and the specific site (S_1 or S_2) as follows: L^S, L^{S1}, L^{S2}, $L^{S1/S2}$, $L^{S/C}$, L^C, and L^X. As shown in the figure, each ligand binds to its own binding site(s). For example, L^S is a ligand that can bind to the specific site of both targets (T_α and T_β), while L^{S1} and L^{S2} bind to the S_1 or S_2 sites only, respectively. This result indicates that the same site can be recognized differently depending on a ligand. $L^{S1/S2}$ binds to both S_1 in T_α and S_2 in T_β. $L^{S/C}$ binds to both site S (i.e., S_1 and S_2) and site C. L^C binds to the common site of T_α and T_β. L^X does not bind to either T_α or T_β.



	Library size	Binding time	Partitioning wash
n1	1-fold	8-fold	4-times
n2	2-fold	4-fold	3-times
n3	4-fold	2-fold	2-times
n4	8-fold	1-fold	1-time

Figure 5. Schematic drawing of SELCOS (or Competitive non-SELEX). The experimental scheme adopted is shown here: 4 successive elution steps that gradually increased the concentration of the library components (1-fold to 8-fold) at the same time, gradually decreasing the washing rounds. Both of these operations favor binding whereas a concomitant decrease in the binding time is unfavorable for binding. The experimental details are described in Methods. In this figure, the competition for the 4 targets is illustrated (a 2- target competition was used in the experiment) since a multiplex type is theoretically more general. After 4 steps of partitioning without PCR amplification, a negative selection (or counter selection) is performed at the final stage.

Estimation of selected ligands by gel-electrophoresis and evaluation by SPR

Surface plasmon resonance or SPR has been extensively used to monitor binding events between analyte and ligand molecules. Thus, utilizing the similar approach for our study we compared the SPR analysis of selected pools obtained by mode of SELCOS for analyte H1N1 and H3N2 with the PCR-amplified selected pool of four round SELEX for analyte H1N1. Fundamentally, we aim to compare the enrichment of the pool selected by SELCOS in presence of two targets with the pool selected by PCR-based SELEX for one target (Figure 7). Our observation suggested that the response value generated against ligand T_{H1N1} for random library was 16 RU which is negligible when compared to the SELEX pool showing 809 RU and 2.99×10^{-8}M KD which exhibits enrichment to certain extent compared with library. However, to our astonishment the SELCOS pool for ligand T_{H1N1} showed a relative higher response value of 2651 RU and 1.01×10^{-10}M KD. Specificity being an important aspect, we decided to check the analyte pool selected for ligand T_{H3N2} by SELCOS against ligand H1N1 and observed a response of 321 RU and 1.99×10^{-7}M KD, relatively lower thus indicating specificity of the selected pool by SELCOS. Henceforth, the preliminary findings from the SPR data were supportive of our theoretical understanding of SELCO mode of action. The above-mentioned observations were determining and motivating to proceed with further analysis of candidate aptamers selected via SELCO.

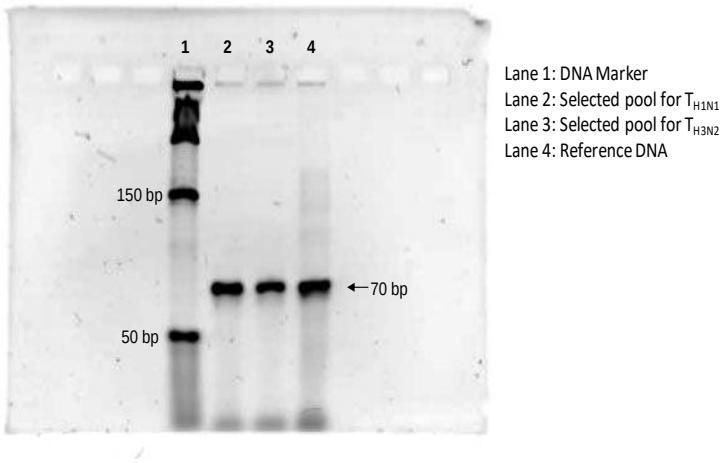


Figure 6. Confirmation of SELCOS products by gel electrophoresis. Electrophoresis was performed under denaturing conditions (8% polyacrylamide gel, 8 M urea at 60 ℃). Obviously, the experimental products with no PCR can result in the same-sized products after selection.

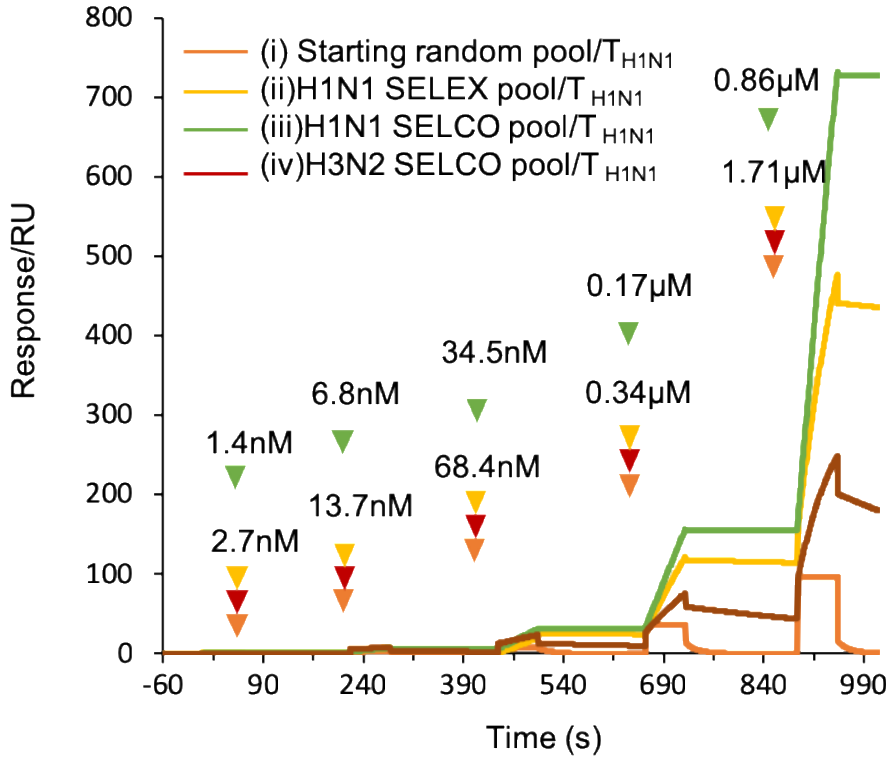


Figure 7. SPR analysis of the selection products with ligand TH1N1. Selected aptamer DNA 5 pools were anlayzed by single-cycle kinetics SPR using a BiacoreX100. For DNA pools, a successive injection of five increasing concentrations (0.0299, 0.149, 0.746, 3.73, and 18.66 μg/mL for analyte sample (i) and 0.0592, 0.296, 1.48, 7.4, and 37 μg/mL for analyte samples (ii), (iii), and (iv) were used. The target protein binding capacity on the sensor chip surface was in levels of 2500-3000 RU (response unit). The X-axis and Y-axis represent the response (RU) and time (s) of the single-cycle kinetics sensogram, respectively. The sensograms were obtained by fitting the data using a 1:1 binding model (BioEvaluation software).

Table 5. SPR analysis on the binding of SELCO products and the target protein used for the selection (T_{H1N1}). 'Pool' indicates a set of DNA aptamers that were just selected. A single cycle kinetics analysis was adopted for the SPR (surface plasmon resonance).

Ligand/Target	**k_{on} ($M^{-1}s^{-1}$)**	**K_{off} ($M^{-1}s^{-1}$)**	**K_D (M)**	**R_{max} (RU)**
Random ligand pool/T_{H1N1}	not sufficiently bound			16
SELEX pool for T_{H1N1}/T_{H1N1}	6.30×10^{3}	1.89×10^{-4}	2.99×10^{-8}	809
SELCO pool for T_{H1N1}/T_{H1N1}	9.34×10^{3}	9.41×10^{-7}	1.01×10^{-10}	2651
SELCO pool for T_{H3N2}/T_{H1N1}	9.06×10^{3}	1.80×10^{-3}	1.99×10^{-7}	321

Evaluation of electrochemical measurements by Apta-DEPSOR

To monitor the quality of the products rapidly, we introduced a DEPSOR-mode electrochemical sensing component (Apta-DEPSOR: see Figure 8). Using two subtypes of influenza A virus as targets, we performed an entire SELCO procedure and monitored the products with the Apta-DEPSOR. As in Figure 9, the products thus obtained (and confirmed in Figure 6) provided the DPV response curves (Panel a) and the corresponding bar charts (Panel b) for the combination of targets (T_{H1N1} and T_{H3N2}) and ligands (ligand pools against T_{H1N1} and against T_{H3N2}), showing that this approach can measure the relative binding strength: the proper matching of a target and a ligand pool provided a far higher signal than those of improper matching, indicating that both SELCO and Electrochemical are working sufficiently well. As described in Methods, the electrochemical sensing is very simple, and this integrated method is very promising for rapid and selective aptamer selection.

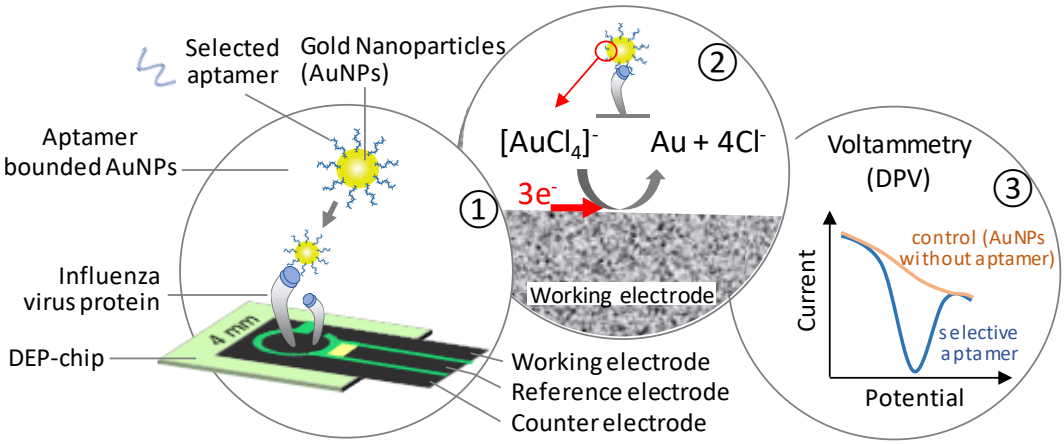


Figure 8. A schematic drawing of the event on the Apta-DEPSOR electrode (Aptamer-based Disposable Electrochemical Printed Sensor) in which the anti-target (influenza virus protein)-DNA aptamer-coated gold nanoparticles (AuNP) bind to the target loaded onto the working electrode of the sensor chip, followed by the electron transfer between the AuNP and the sensor surface, resulting in the generation of the DPV (differential pulse voltammetry) pattern.

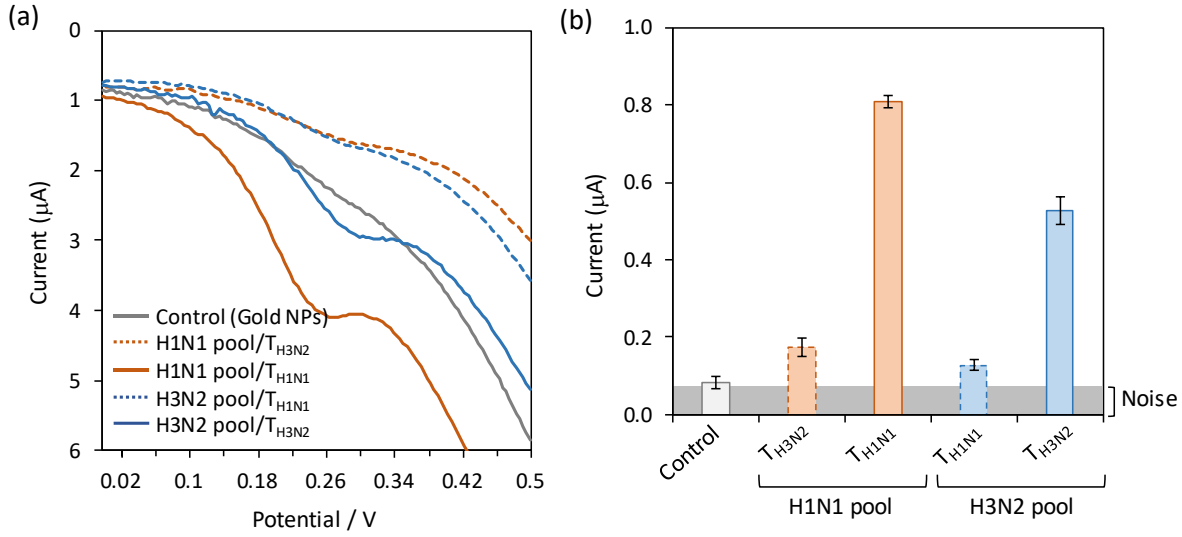


Figure 9. SELCO products. Aptamer pools obtained against T_{H1N1} (i.e., target H1N1, in red) and T_{H3N2} (blue) were subjected to the electrochemical measurement using Apta-DEPSOR. (a) For each sample, the DPV was measured against both T_{H1N1} and T_{H3N2}. (b) The I_{pc} (current for the signal peak) data are presented in a bar chart (using the average taken from 3 independent experiments).

Theoretical Note

The reason that SELCO is superior at finding target-selective aptamers relative to conventional SELEX is discussed in the following theoretical note. To explain the phenomenon that occurs in the competitive non-SELEX selection (SELCO), a pool of ligands can be categorized into 7 types from the perspective of their binding to one or multiple target molecules: L^{S}, L^{S1}, L^{S2}, $L^{S1/S2}$, L^{C}, $L^{S/C}$ and L^{X}. Each symbol represents a ligand that binds to common site C of two targets of T_α and T_β (L^{C}), a ligand binding to specific site S_1 within T_α (L^{S1}), a ligand binding to specific site S_2 within T_β (L^{S2}), ligands binding to both sites S_1 and S_2 simultaneously (L^{S} and $L^{S1/2}$), a ligand that binds to both targets T_α and T_β ($L^{S/C}$) and the other ligands, which do not bind to either T_α or T_β (L^{X}). This setup can be represented by the following equations based on conservation law:

$$[L^{S1}\cdot S_1]+[L^{S1/S2}\cdot S_1]+S_1 = [S_1]_0 \quad (1)$$

$$[L^{S2}\cdot S2]+[L^{S1/S2}\cdot S_2]+S_2 = [S_2]_0 \quad (2)$$

$$[L^{C}\cdot C]+C = [C]_0 \quad (3)$$

$$[L^{S1}\cdot S_1]+[L^{S1}] = [L^{S1}]_0 \quad (4)$$

$$[L^{S2}\cdot S_2]+[L^{S2}]= [L^{S2}]_0 \quad (5)$$

$$[L^{C}\cdot C]+[L^{C}]= [L^{C}]_0 \quad (6)$$

$$[L^{X}] = [L^{X}]_0 \quad (7)$$

$$[L^{S1/S2}\cdot S_1]+[L^{S1/S2}\cdot S_2]+[L^{S1/S2}] = [L^{S1/S2}]_0 \quad (8)$$

$$[L^{C}]+ [L^{S1}]+[L^{S2}]+ [L^{S1/S2}]+ [L^{X}]+ [L^{C}\cdot C] + [S_1]_0 + [S_2]_0 = \sum L = [L_0] \quad (9)$$

where $[S_1]_0$ and $[S_2]_0$ designate the initial concentrations of sites S_1 and S_2, which are equal to $[T_\alpha]_0$ (that is, the initial concentration of the target T_α) and $[T_\beta]_0$ (that of the target T_β), respectively, and L_0 stands for the initial ensemble concentrations of ligand L. Similarly, C and $[C]_0$ represent the concentration of the common binding site and its initial one, and thus,

$$[C]_0 =[T_\alpha]_0 +[T_\beta]_0 \quad (10)$$

For convenience, we can set $[T_\alpha]_0 =[T_\beta]_0 =T_0$ operationally. Then,

$$[C]_0 = 2\cdot T_0 \quad (11)$$

This result indicates that when the initial concentration of L^{C} is the same under SELCO and conventional positive SELEX, the concentration of $L^{C}\cdot T_\alpha$ for Competitive non-SELEX becomes half of that for the conventional form due to the following equation.

$$L^{C}\cdot C = L^{C}\cdot(T_\alpha + T_\beta) = 2L^{C}\cdot T_\alpha \quad (12)$$

Since L^C is a nonspecific binder (aptamer), the SELCO can thus reduce the nonspecific aptamers through a 'nonspecific target multiplying' effect. This effect can be further reinforced by multiplying the diversity of competitors in SELCO as in the case of influenza subclass viruses.

Another effect of SELCO for selecting more specific aptamers than the conventional method (typically, a combination of negative selection and positive selection) is shown in T_α) can be determined from the following Eqs. 13, 14, and 15 as derived from Eqs. 1, 2, and 8:

$$[L^{S1/S2}\cdot S_1] = [S_1]_0 - [L^{S1}\cdot S_1] - S_1 \quad (13)$$

$$[L^{S1/S2}\cdot S_2] = [S_2]_0 - [L^{S2}\cdot S_2] - S_2 \quad (14)$$

$$[L^{S1/S2}\cdot S_1] = [L^{S1/S2}]_0 - [L^{S1/S2}\cdot S_2] - [L^{S1/S2}] \quad (15)$$

Clearly, in the case of noncompetitive SELEX, the terms related to S2 located in T_β (not applied) do not appear. Now, Eq. 15 is converted to

$$[L^{S1/S2}\cdot S_1] = [L^{S1/S2}]_0 - [L^{S1/S2}] \quad (16)$$

The left-hand side is evidently larger by $L^{S1/S2\cdot S2}$ than that of SELCO, meaning that SELCO can decrease the amount of nonspecific aptamer ($L^{S1/S2}$) by this effect. Even so, Eqs. 13 and 14 are noteworthy. Eq. 13 represents a typical competition between different ligands competing for the same site while Eq. 14 represents latent competitor L^{S2} (i.e., how strong it is in struggling for site S_2), which has an indirect influence on the recovered amount of target aptamer L^{S1}.

The above estimation must be carefully applied under the premise of dealing with nonextreme conditions (i.e., excluding high excess concentrations of the targets ($T_0 \gg L_0$) or ligands ($T_0 \ll L_0$) so that the competitions of interest are working well). A more quantitative and parametrical approach, although not presented here, will be possible, as in the previous work.

4. Conclusion

The theoretical description of the variation in the behavior of the ligand molecules in competitive selection presented with a proof-of-concept with experimental details of kinetic comparison of selected pool via PCR-free and competitive selection (two target) and single target PCR selection, supported the novel concept of competitive selection. Also, the electrochemical measurements for evaluation of specificity of the selected pool of ligands via SELCO highlight the selection of highly specific ligands. Thus, the preliminary findings prove supportive and motivating for the identification of the selected ligands.

Chapter III Identification and evaluation of selected aptamers via SELCO for subtype H1N1 and H3N2 of Influenza A virus

1. Introduction

Nucleic acid aptamers find widespread use as targeting and sensing agents in nature and biotechnology. The aptamer characterization platform is broadly useful for studying aptamer–ligand interactions, comparing aptamer properties, screening functional aptamers during *in vitro* selection processes and prototyping aptamers for integration into nucleic acid devices[117]. Aptamers screened via *in vitro* selection yield a pool of candidates however not all the nucleotides contribute to the high binding affinity for the target[118]. An aptamer can be divided into three regions: a region of essential nucleotides, supporting nucleotides, and nonessential nucleotides. The essential region consists of nucleotides that are directly involved in the interaction of the aptamer with the target. Any base substitutions or removal of a base in this region will result in a significant loss of binding affinity. The second region consists of supporting nucleotides. Supporting nucleotides indirectly contribute to the binding affinity by stabilizing the secondary structure of the aptamer. Stabilization occurs through the formation of stems by intramolecular base pairing of complementary nucleotides. Base substitutions or decreases in the stem length result in modest decreases of the binding affinity. The third region is the nonessential region and consists of nucleotides that can be substituted or removed without a loss in the binding affinity for the target. It typically consists of nucleotides that do not participate in intra- or intermolecular binding[119–122].

Clustering and alignment methods allow the analysis of the frequency distribution of sequences in the data set and the formation of sequence families. In addition to sequence similarities, consensus motifs based on common secondary structures can be analysed through modelling software[123,124]. The secondary structure of the aptamer is important in binding the target because various folds allow the aptamer to exploit various binding mechanisms such as hydrophobicity, molecular shape complementarity, or intercalation in the case of small molecules binding double-stranded regions of the aptamer. To identify common folds or secondary structures, computer programs have been developed to predict

the secondary structures based on minimizing free energy using thermodynamic data, which can then be used to compare aptamer structures[125–127] The inclusion of structure prediction tools gives information about the enrichment of essential structural motifs. Finally, the impact of different selection conditions on the outcome of an experiment can be studied more easily. The selection process generally results in a large number of potent aptameric sequences, which need to be evaluated individually for their binding to the target. The most promising sequences are then chosen as candidate aptamers for an extensive characterization in terms of their target-binding affinities and specificities, their structural features including sequence optimizations and modifications, and their functionalities under different binding conditions.

This is a very costly and time-consuming process, but absolutely necessary for the identification of the best and most suitable aptamer and hence for increasing the acceptance of aptamers and bringing them into real applications. In this chapter, sequence analysis by clustering and predictable secondary structure of the candidate aptamers selected via SELCO for subtype H1N1 and H3N2 of Influenza A virus is described. Also, the kinetic and electrochemical properties are studied.

2. Materials and Methods

Cloning and Sequencing

The details of the cloning and candidate determination are mentioned as following. pCR2.1 TOPO vector (TOPO TA Cloning Kit, Invitrogen) has been used for cloning and transformed into TOP10F` competent E. coli cells. Ampicillin was purchased from Sigma-Aldrich (St.Louis, USA) and X-Gal (5-Bromo-4-chloro-3-indolyl-ß-D -galactopyranoside) was purchased from Roche Diagnostics (Mannheim, Germany). In brief, all the selected pools were then cloned, and 20 clones (blue-white screening) were picked for sequencing (Eurofins, Tokyo, Japan). Positive transformants were analyzed by gel-electrophoresis. Sequence analyses were performed using the web-based tools ClustalW[123] and Mfold[128], for multiple sequence alignment (for details, see Figure 10) and secondary structure analysis, respectively.

SPR Measurements

To study the interaction analysis (association/dissociation) of candidate aptamers selected via SELCO, single-cycle mode was performed for the immobilization of the ligand-target protein H1N1 with the his-tag (0.01 mg/mL) in the running buffer onto the sensor surface at a level of 2000 RU, with a contact time of 120 s and a stabilization period of 60 s. Aptamer solutions of the following concentrations: 26.66, 5.33, 1.07, 0.213, and 0.0427 µg/mL were prepared in the running buffer and sequentially injected, starting with lowest concentration, at a flow rate of 30 µL/min for 60 s, followed by 60 s of dissociation. All measurements of the binding analysis were performed in triplicate and the fitting was done for the 1:1 binding model by BiacoreX100 Evaluation software. The single-cycle mode was performed for the immobilization of the ligand protein H3N2 (0.01 mg/mL) in the running buffer onto the sensor surface at a level of 1000 RU, with a contact time of 120 s and stabilization period of 60 s. Aptamer solutions of the following concentrations: 44.44, 8.89, 1.78, 0.356 and 0.0711 µg/mL were prepared in the running buffer; similar conditions for injection were used and the resulting binding curves were studied.

Electrochemical Measurements

Please see the Method section in Chapter II. The same protocol and experimental conditions have been maintained for this analysis for uniformity and check the response for the selected ligands from the same pool tested before for the respective targets.

3. Results and Discussion

Evaluation of cloned DNA aptamers

After SELCO was performed with the targets T_{H1N1} and T_{H3N2}, the selected pools were subjected to cloning and sequencing, providing multiple aptamers (Figure 10) with some of representative aptamers listed in Table 6. Interestingly, the secondary structures of aptamers selected against the target H1N1 (i.e., Apt01~Apt04>T_{H1N1}) have a common motif of 'Loop1-space-Loop2' in which common sequences are involved (GGTCAG in Loop1 and T(or C)T(or A)GT in Loop2, although the GGTCAG sequence happens to come from the primer binding site), while the aptamers selected against T_{H3N2} have no similarly remarkable characteristics as far as the evidence shows (partly shown in Figure 11). These conserved loop regions (Loop1 and Loop2) are highly expected to interact with the target molecules[129,130] Although it is a much simpler and more rapid method than conventional SELEX, SELCO can attain to, sometimes, find putatively functional motif as shown here. The ΔG values for aptamer folding are shown in Table 6, and they present moderate stability values ranging from -6 to -12 kcal/mol with no significant correlation with the affinity K_D (r=-0.11). In Table, it is noteworthy that the frequency score that appeared for each selection has a relatively high correlation value (r=0.55) with the binding affinity of K_D, conforming to a rule that 'the higher the affinity is, the higher the population'.

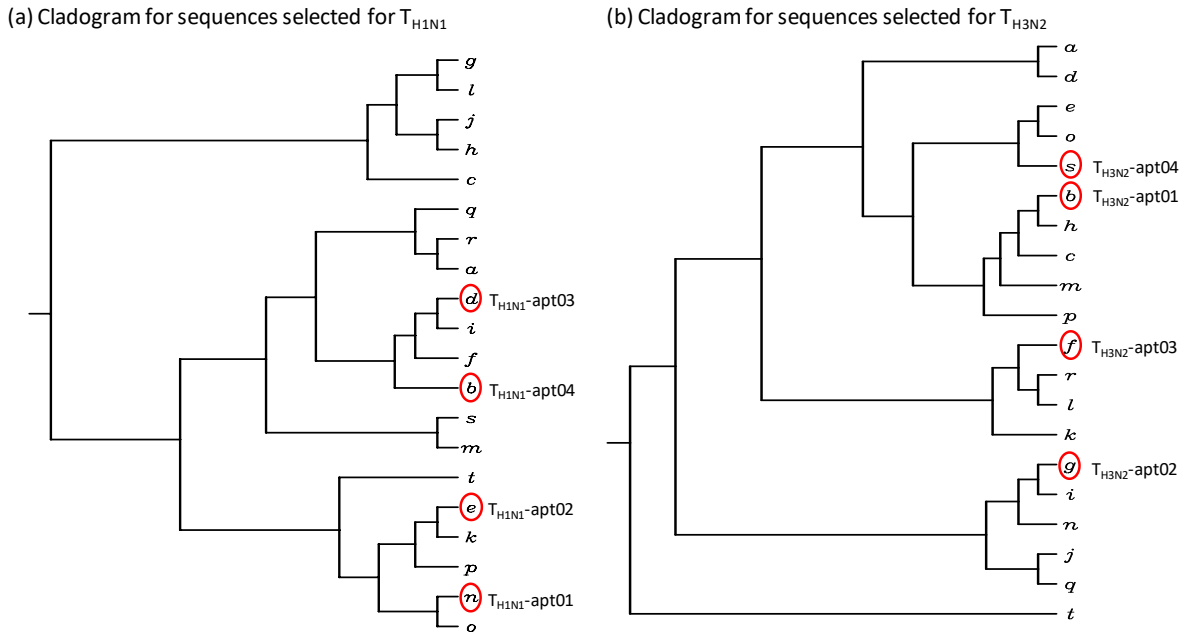


Figure 10. Cladograms generated for aptamer sequences obtained by Compe-SELEX targeting H1N1 and H3N2 proteins. The encircled aptamers in these cladograms are renamed (parenthesis) and processed for further analysis. (Ref.: for Clustal W-based cladogram analysis: Thompson et al., *Nucleic Acids Res*. 1994, 22, 4673. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice).

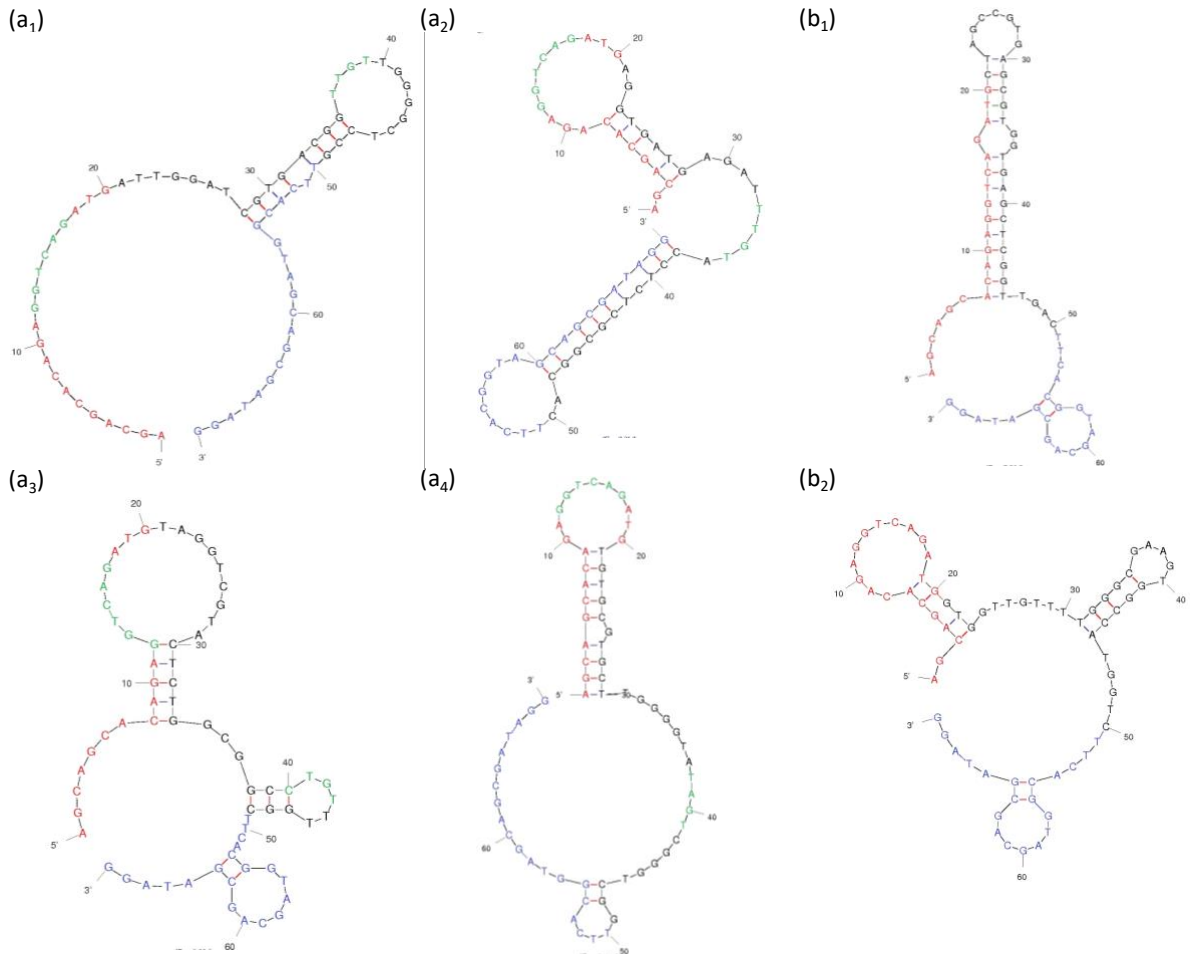


Figure 11. Predicted secondary structures of cloned aptamers. Some of the aptamers obtained against T_{H1N1} and T_{H3N2} were analyzed with Mfold, a secondary structure-computing program. (a1-a4) For the aptamers selected against T_{H1N1} (namely, Apt01>T_{H1N1}, Apt02>T_{H1N1}, Apt03>T_{H1N1}, Apt04>T_{H1N1}). (b1 and b2) For the aptamers Apt01>T_{H3N2} and Apto2>T_{H3N2}. Commonly appearing sequences in loop regions are highlighted for the aptamers against T_{H1N1} (incidentally, no such sequences were found in the aptamers obtained against T_{H3N2}). Note that the sequence regions of 1-20 and 51-70 over the entire sequence (70 nucleotides) are primer-binding sites, and they are constant.

Evaluation of SPR measurements of DNA aptamers

As shown in Figure, from the kinetics analysis with SPR (Biacore X100) when employing the single cycle mode analysis, data in Table were obtained. Under our experimental conditions, the selected aptamer pool against T_{H1N1} in the SELCO had a 300-fold stronger K_D than the value selected by conventional SELEX, and this K_D (1.01×10^{-10} M) is already close to that of the cloned aptamer Apt03>T_{H1N1} (0.82×10^{-10} M). The Apt03>T_{H1N1} aptamer is more than 100-fold stronger than that of the previously reported DNA aptamer RHA0006[131] that was selected against influenza virus subtype H1N1. For reference, the commercial monoclonal antibody was also measured by SPR, showing the strongest affinity (2.53×10^{-13} M), which was surprisingly sophisticated. Interestingly, the Apt03>T_{H1N1} aptamer exhibited a similar pattern of fitted curve, showing a high resemblance of the calculated kinetic parameters (for details, see Figure 12. This important data addresses some key aspects for future approaches involving the replacement of antibodies with aptamers. In any case, SELCO provided a sufficiently competent aptamer in terms of its binding affinity.

Ligand	k_{on} ($M^{-1}s^{-1}$)	k_{off} ($M^{-1}s^{-1}$)	K_D (M)
mAb	2.331×10^{5}	5.903×10^{-8}	0.253×10^{-12}
apta03	3.736×10^{4}	3.075×10^{-6}	82.34×10^{-12}

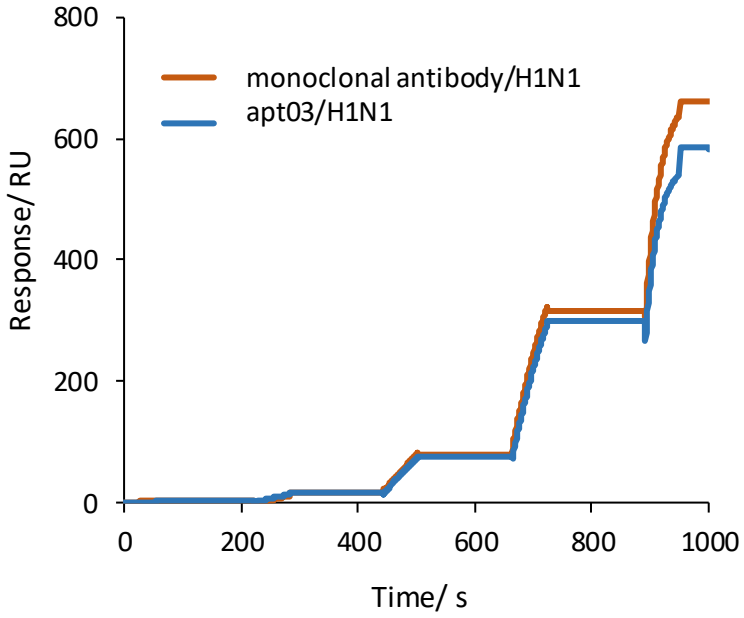


Figure 12. SPR analysis of selected aptamer vs. antibody. Comparison of selected aptamer Apt03>T_{H1N1} by SELCOS (blue) with monoclonal antibody (orange) for target H1N1 showing comparable kinetics. Single-cycle kinetics conditions for the study of interaction analysis used are as follows: Immobilization of ligand-target protein H1N1 on the sensor surface to level 2000 RU, Analyte (Apt03>T_{H1N1} and mAb) solutions were prepared in the order 26.66, 5.33, 1.07, 0.213 and 0.0427 µg/ml and sequentially injected starting with lowest concentration at a flow rate of 30 µl/min and the fitting was done by 1:1 binding model by BiacoreX100 Evaluation software.

Evaluation of electrochemical measurements of DNA aptamers

These aptamers were electrochemically analyzed separately as shown in Figure 13. The binding strength to its original target was tested by SPR for these aptamers (Table 6), for which the I_{pc} values obtained from the electrochemical method are also shown (giving a correlation score of -0.40 with the K_D measured by SPR). From this result, the I_{pc} value can be used to estimate the binding strength of aptamers though less exactly.

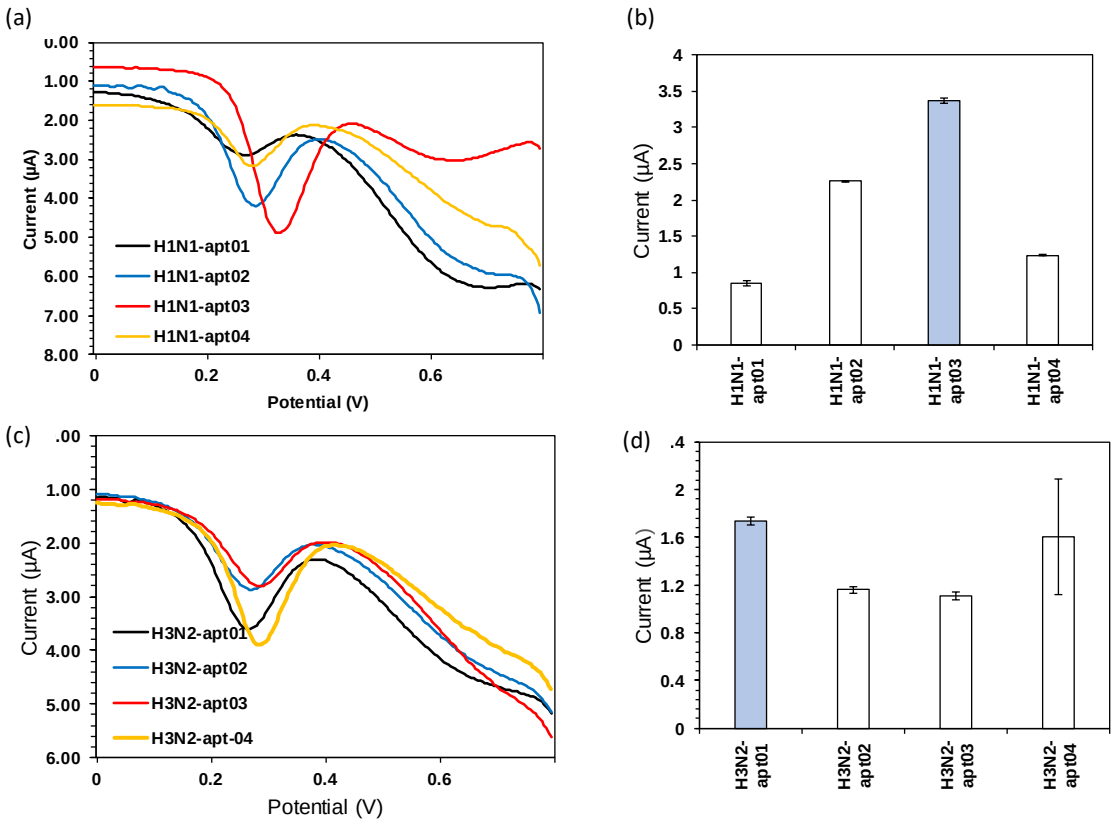


Figure 13. Measuring the binding affinities of cloned aptamers. The DNA aptamers selected by SELCOS were subjected to Apta-DEPSOR analysis using the corresponding targets, providing the DPV curves (a and c) and its processed data (b and d). The aptamers Apt01~Apt04>T_{H1N1} were measured against the target H1N1 protein (T_{H1N1}) (a and b) and the aptamers Apt01~Apt04>T_{H3N2} against T_{H3N2} protein (c and d). In b and d, the average data for three trials are plotted.

Table 6. Properties of the aptamer DNAs selected against influenza virus proteins obtained by SELCOS. Aptamer DNAs were obtained from a single trial of SELCOS that offered two sets of aptamers, T_{H1N1}-specific and T_{H3N2}-specific ones. The ones listed here were chosen by the clustering analysis. The sequence frequency of appearance of the aptamer within a selected DNA pool, free energy for folding, dissociation constant (K_D) for the binding of the target protein and aptamer, and peak current for the signal (I_{pc}) in the electrochemical analysis are listed.

ID for cloned aptamer[α]	Aptamer sequences[β] (5`---3`)	Frequency of appearance (%)	ΔG[χ] (kcal/mol)	KD[δ] (M)	Ipc[ε] (μA)
Apt03>T_{H1N1}	5'PBS-TAGGTCGTAC TCTGGCGGCC TGTTTGGC-3'PBS	8.33	-6.32	0.82×10^{-10}	3.37 ± 0.035
Apt04>T_{H1N1}	5'PBS-TGTGCGTGCT TGGGGTATAG TCGGGTCGG-3'PBS	4.17	-5.96	0.16×10^{-8}	1.23 ±0.011
Apt02>T_{H1N1}	5'PBS-AGGTGATGAG ATTTGTACCT CTCGCGGCAC-3'PBS	8.33	-9.31	0.57×10^{-7}	2.26 ±0.011
Apt01>T_{H1N1}	5'PBS-ATTGGATCGT GACGGTTGTT GGGGCTCCG-3'PBS	12.5	-5.33	0.35×10^{-4}	0.85 ± 0.036
Apt04>T_{H3N2}	5'PBS-TCTGCAGCGT GCAGGGCTGT GTGCTTACCC-3'PBS	4.17	-9.83	0.88×10^{-10}	1.61 ± 0.48
Apt01>T_{H3N2}	5'PBS-CTAGCCGTGA GCGTGGTGAG CTCGGTTGAC-3'PBS	12.5	-7.51	0.14×10^{-9}	1.73 ± 0.032
Apt03>T_{H3N2}	5'PBS-GCGCGGGCGG TGCGTCGGTG TCCCGCTGG-3'PBS	4.17	-12.50	0.60×10^{-9}	1.11 ± 0.032
Apt02>T_{H3N2}	5'PBS-GTGGTTGTTT TGGGCGAAGT GGCCATGGTC -3'PBS	8.33	-5.51	0.17×10^{-8}	1.16 ± 0.026

[α]Nomenclature for cloned aptamers were systematically assigned to be 'serial#' (e.g., Apt01) + '>' (a connector) + 'Target name' (e.g., T_{H1N1}), thus Apt01>T_{H1N1}

[β]5'-PBS and 3'-PBS are primer binding sequences (AGCAGCACAG AGGTCAGATG and CCTATCGCTG CTACCGTGAA, respectively)

[χ]dG (kcal/mol) is free energy value and calculated from Mfold online tool

[δ]KD (M) value is generated by BIACORE X100 using single cycle kinetics

[ε]I_{pc} (μA) is average current value and obtained from DPV curves

Discussion (SELCO)

Competitive non-SELEX (SELCO) was shown to be effective at enriching aptamers for which evaluations can be empowered by the results. Here, we further deepen this explanation in comparison to the conventional SELEX. The typical difference between these two methods relates to whether multiple targets can coexist or not. The current SELCO application was operated under the experimental conditions described in Methods. There were 4 steps of imposed selection pressure (a step-wise reduction in the binding time during the increase in binding ligands with a gradual weakening from the washing effect), the successive addition of a ligand library (without PCR amplification) and one step of negative selection at the final stage. Originally, SELEX (Systematic Evolution of Ligands by EXponential enrichment) had the following essential properties: it employs an RNA/DNA library; i) under selection pressures with increasing stringency, ii) it partitions materials into two groups that are 'binding' and 'nonbinding' (usually 'solid phase of beads or resin' and 'bulk solution'), and iii) it involves the amplification of the library (usually by PCR). SELCO has a different mode than conventional SELEX as the intermediate amplification step, i.e., PCR, of conventional SELEX is excluded in SELCO and addition of stock supply from the original library was used between repetitive steps of partitioning. However, we must pay careful attention to the difference in the PCR amplification step of conventional SELEX and stock supply of SELCO. First, we would like to point out that the naming of SELEX has been successful due to the appealing nature of its methodological details and the term´s nice compact ring. We have no doubt that it was a wonderful invention in the field of molecular evolution[132]. However, the words contained in "SELEX" might have been confusing since "exponential enrichment" is not correct if one believes that the molecules to be selected are enriched in an exponential mode; although the library components are amplified exponentially by PCR, no relative enrichment of a particular element occurs during this exponential amplification stage. The PCR amplification of a pool of ligands is expressed as shown below.

$$L_i(t) = L_i(0)\cdot(1+e\,(t))^t \qquad (0 \le e\,(t) \le 1) \tag{1}$$

$$L_1(t) / L_2(t) = L_1(0)\cdot(1+e\,(t))^t / L_2(0)\cdot(1+e\,(t))^t \tag{2}$$

$$= L_1(0)\cdot/ L_2(0) = \text{const.} \tag{3}$$

where $L_i(t)$, $L_1(t)$, and $L_2(t)$ represent the concentrations of ligands L_i, L_1, and L_2 at time t, respectively, and $L_i(0)$, $L_1(0)$, and $L_2(0)$ are the ligand concentrations at time 0, and they are

constant. The letter *e*(t) signifies the amplification efficiency at time t (the time is the integer). In PCR, ideally, *e*=1. However, this value usually depends on the time due to the inactivation of polymerase, the decrease in primers, the increase in reactants, and so on. Since these factors are commonly influential for each template, the same *e*(t) value can be expected for each template. Thus, Eq. 3 holds true, indicating that the PCR does not change the ratio of constitutive elements before and after the PCR. In other words, no enrichment occurs during PCR, but the simple amplification of each component by the same factor (i.e., $\cdot(1+e(t))^t$) does lead to enrichment. Notably, PCR is well-known for generating alterations in the population of a library; the ratio changes in the population and the mutation of DNA/RNA have no relation to the enrichment of the fitting aptamers. Parameter *e* also depends on the template DNA/RNA itself since these nucleic acids generate secondary and tertiary structures intrinsic to their sequence, and those structures are often unfavorable for the polymerization reaction (thus lessening the parameter *e* value). This effect can generate a bias in the population, but it does not denote an enrichment of the fitting aptamers; the axis of selection is quite different. Therefore, "exponential enrichment" is image-inducing wording but it is not realistic. However, since PCR, a typical process of SELEX, is not included in our method, we adopted the name "SELCO" for our technology.

The reason (Theoretical Note) that SELCO is superior at finding target-selective aptamers relative to conventional SELEX is discussed in the theoretical note. In brief, SELCO can find more target-selective aptamers (L^{S1} and L^{S2} drawn in Figure.) due to the competitive effect of the ligand-binding between target molecules (T_α and T_β). To be sure to obtain this effect, a thermodynamic equilibrium must be attained. In SELEX, successive subtraction is repeated by washing (partitioning) to enrich the aptamers of interest. During this process, due to the kinetic effect, a large population of fitting aptamers (such as L^{S1} and L^{S2}) could be irreversibly lost by washing, which can be effectively circumvented by SELCO due to the successive addition of the entire population of ligands. This approach can rationalize the experimental results in which SELCO succeeded in yielding a higher affinity ligand pool than SELEX, as shown in Table (although using a computer simulation that assumes a set of parameters might be more persuasive[133]). At the same time, the effect decreases the relative number of ligands ($L^{S/C}$ and L^C in Figure) since they bind common sites, which is multiplied when there are multiple targets, which must also contribute to the relative enrichment of target-selective

aptamers. These effects are usually obtainable when the negative selection (a selection that eliminates the ligands that bind non-authentic targets) is performed. Thus, SELCO has the ability to provide a negative selection in parallel with a positive selection. This property confers SELCO with cost savings relative to SELEX since it can provide M-multiple different aptamers at once when M-tiple targets are adopted (although in this article, M = 2). Clearly, in such an M-tiple target system, the ultimately selected aptamers can be expected to be exclusively selective for the relevant target with nonbinding with the other targets.

4. Conclusion

The secondary structure of candidate identified for subtype H1N1 depicted similar pattern in stem-loop region thus attractive from the perspective of binding characteristics of aptamer with target biomarkers. The aptamer Apt-03 for subtype H1N1 and Apt-01 for subtype H3N2 showed high binding-affinity in addition to significant signal in electrochemical measurements, detected by aptamer-immobilized on Au-Nano particle indicating the sensing potential of the identified aptamers. Also, Apt-03 pattern of kinetics of association and dissociation resemble to a monoclonal antibody, compared with experiment is significant for application of aptamers as recognition bio-probes.

Chapter IV Development of Apta-DEPSOR for selective point-of-care testing of infective-subtype of Influenza A virus

1. Introduction

The biosensor research, based on interdisciplinary fields of chemistry, biology and engineering, developed mainly due to the requirement for fast and reproducible/reliable on-site medical diagnosis in the absence of specialized human and technical resources. Immunosensor biosensors were especially developed on the fact that antibodies (proteins) specifically bind towards their respective antigens with high affinity and the transducer detects the immunochemical reactions directly or indirectly. Immunosensors developed continuously from the perspective of better sensitivity and selectivity to meet the requirement of detection of antibody or antigen in body fluids for the timely detection of disease. However, the conventional methodologies demanded relatively large volumes of sample, long incubation periods, complicated apparatus and highly trained laboratory staff. Other than these obvious limitations that could be overcome, there were some specific problems related to the immobilization and orientation of the large size antibodies which was quite challenging. It was preferred not to immobilize the antibodies thus allowing for free binding sites on the antibody surface and also multiple labeling strategies needed to be developed that would not interfere with the antigen-antibody interactions. Other than this the antibody pool was not well developed for a wide range of targets including low molecular weight/small biomarkers such as peptidome. Henceforth, keeping in view the origin of immunosensors for utility in rapid analysis of epidemic wide spread, proper diagnosis of unreachable target biomarkers and to be able to perform multiple assays where conventional methodologies such as ELISA failed, researchers focus on aptamers for detection strategies as the properties of aptamers offer a promising alternative to be able to meet the current demands and the limitations created by the challenges posed by antibodies in the sensing technology. Substantial progress has been made in the development aptamer-based sensors for the diagnostic/detection purposes. Aptasensors are biosensors based on aptamers as recognition element. The Biyani group has been actively engaged in developing the platform of electrochemical sensing via the smart tools of rapid sensing technology. DEP-Chip series, DEPSOR-M are the trade name of some of their products developed for the fabrication of

various biosensors, manufactured under the startups Biyani Biosolutions Pvt. Ltd. (India) and BioDevice Technology, Ltd. (Japan). These companies mainly focused on the development of biosensors having the potential to meet the need of inexpensive, rapid and hand-held systems for the benefits of society triggering targets relevant to environmental, clinical, food, medical and industrial purposes. The screen-printing technology developed by Ahmed et al 2007 and other groups[134,135] introduced the concept of printing/coating solid electrodes in parallel. This has led to miniaturization of electrochemical immunosensors, termed as disposable screen-printed electrochemical (DEP) chip. The DEP chip sensors are easy to use, contamination free and disposable. They are inexpensive from the perspective of production as well as the reagents and sample required in small volume1-2μL. This technology is high-speed, reproducible and cost-effective, making possible to detect unreachable target analytes/biomarkers. The idea of GLEIA (Gold-Linked Electrochemical Immuno-Assay) (PCT/JP2006/56992) was developed for the detection of antigens via redox signals generated by the gold nanoparticle. After the recognition reaction between the surface-immobilized primary antibody and hCG, the captured antigen was sandwiched with a secondary antibody that was labeled with gold-nanoparticles. Then the amount of gold nanoparticles was determined by its redox signal[136]. The highlight of this concept when compared to ELISA was that electrochemical measurement was used thus improving the limitations of optical based measurement. And the analysis time was reduced to less than the half time required for ELISA based approach and the required sample volume was 1/10 th of the conventional approach, as little as 1 μL. In 2015, Biyani et al. group merged the DEP chip technology with the in vitro selection approach and introduced the novel idea of PEP-on-DEP i.e.; firstly, the peptide aptamer was identified for target renin (biomarker for kidney dysfunction and/or hypertension) using advanced evolutionary molecular engineering as reported previously by Kitamura et al., 2011 and further integrated the peptide onto the surface of a screen printed-DEP chip. In this chapter, the development of a more practical aptamer based immunosensor for emerging infective strains has been highlighted. The principle of generation and characterization of novel probes, merging with electrochemical transduction platform can be adapted to develop a portable, low-cost and mass-producible biosensor for point of care applications. Such aptamer-based biosensor is essential for the fast-tracking of the molecular diagnostics.

2. Materials and Methods

Electrochemical Measurements

The specificity validation experiments for identified aptamer were performed similarly as mentioned previously. For competitive assay, the protein target was immobilized similar to the protocol mentioned previously. Then, a 2-µL sample made up of Au nanoparticles conjugated to the selected DNA aptamer pool candidates was saturated with different concentration of target protein for the respective aptamer for 15 min at room temperature. Then the saturated complex (target-aptamer-gold colloid) was dropped onto the target-modified DEP chip surface; the chip was then incubated for 15 min at room temperature. It was then rinsed three times with 100 mM PBS buffer and connected to an electrochemical analyzer system (Model 650 A, CH Instruments, Inc., Austin, USA). Then the signals were recorded similar to the experimental conditions mentioned previously. All the experiments were repeated three times to confirm the consistency of the analysis.

3. Results and Discussion

Specificity evaluation of identified aptamer

The aptamer Apt01>T_{H3N2} (denoting an aptamer named apt01 obtained in the selection targeting T_{H3N2}) is shown to have an approximately 9-fold higher current signal (I_{pc}, cathodic peak current) against T_{H3N2} than against T_{H1N1}. Similarly, the Apt03>T_{H1N1} aptamer is more selective for its original T_{H1N1} target (near 5-fold higher current signal) than the nontarget T_{H3N2} (Figure 14).

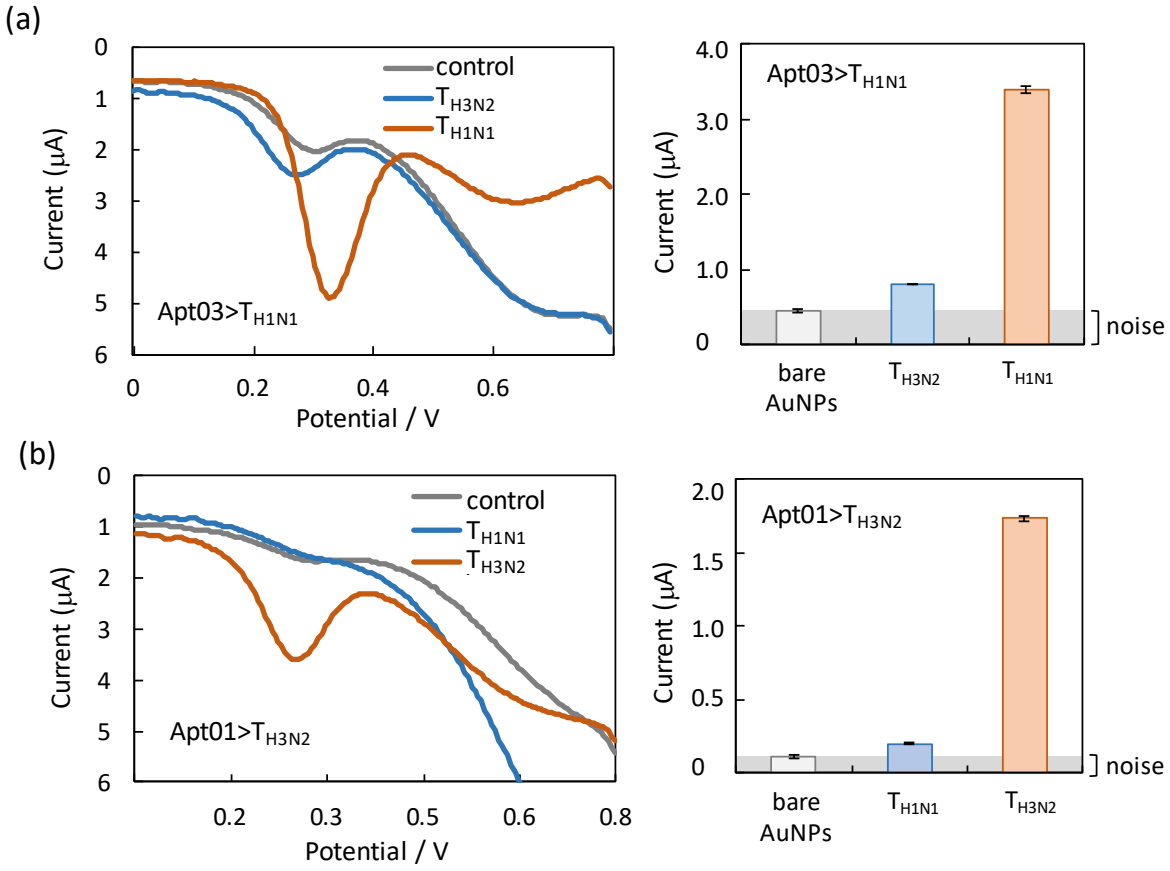


Figure 14. Validation of selected aptamer molecules by Apta-DEPSOR. (a) The aptamer, Apta03>T_{H1N1} (namely, aptamer #03 selected against the target H1N1 protein (T_{H1N1})), was measured against T_{H1N1} and T_{H3N2}. The DPV curves (left) and the corresponding bar graph (right) are shown. (b) The aptamer, Apt01>T_{H3N2}, was used here. "Control" (gray) indicates the signal from bare gold nanoparticles (AuNP). The concentrations of the target proteins, T_{H1N1} and T_{H3N2}, were both 250 μg/mL.

Quantitation of an influenza virus subtype using Apta-DEPSOR

An influenza subtype H1N1-specific aptamer (Apt03>T_{H1N1})-loaded Apta-DEPSOR was fabricated as shown in Figure 15. First, the working electrode surface of a DEP chip was covered with the free target H1N1 protein (T_{H1N1}), and, then, a fixed amount of AuNP (gold nanoparticles) mixed with a virus sample is introduced to the electrode, where, when there is excess AuNP relative to the free T_{H1N1} of virus sample, the excess amount of free AuNP coated with the aptamer (Apt03>T_{H1N1}) can bind to T_{H1N1} on the surface of the electrode and then be trapped and detected by the sensor. In this experimental system, the electrochemical signal will increase depending on the amount of AuNP captured by the T_{H1N1} on the electrode surface, and it will decrease proportionally depending on the amount of target T_{H1N1} protein in the sample solution. Figure. shows the dependence of the DEPSOR signal (current) on the concentration of applied T_{H1N1}. From the resulting calibration curve, the dynamic range of the measurements ranged from 0.4 to 100 μg/mL in 5% human serum and we successfully detected T_{H1N1} in as little as 1.23 ng/L.

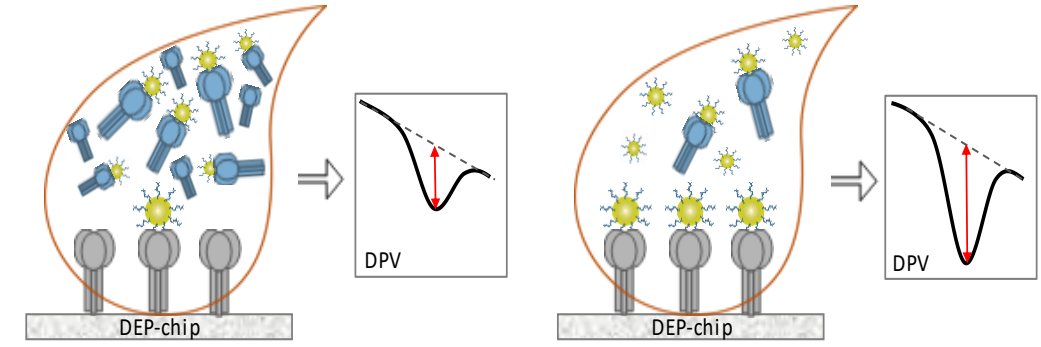


Figure 15. The electrochemical sensing point-of-care application. A virus protein concentration-dependent measurement of the DPV. The AuNPs are carried away with free protein when the flowing sample solution contains a large amount of virus protein. Note that the dent in the DPV curve is the signal in proportion to the bound AuNP.

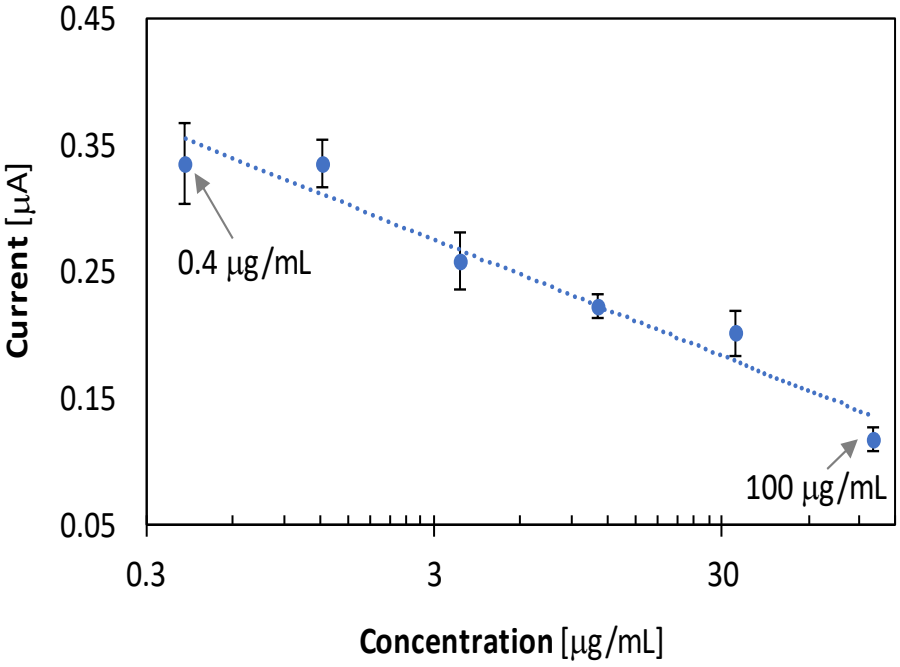


Figure 16. Calibration curve for measuring influenza virus A subtype H1N1 in human serum. A bound-and-free virus (protein) competition assay using Apta-DEPSOR was performed as explained in Fig. 1-b2. In this assay, there is competition for aptamer-coated gold nanoparticles (i.e., T_{H1N1}-apt03-coated AuNP) by two phases of proteins (free and electrode surface-bound). From the regression analysis, the correlation coefficient *r* was -0.88. The lower detection limit was 0.51. The figure shows the averages taken from three trials.

4. Conclusion

Competitive non-SELEX (SELCO) was shown to be effective at enriching aptamers that were specific to a target protein, for which evaluations can be empowered by the introduction of the Apta-DEPSOR, a kind of electrochemical sensing device. Among the various detection devices, such as SPR, Apta-DEPSOR is useful for the current purpose due to its detectability of ligand-target interactions, readiness for operation and portability, and potential use in constructing a POC device for detecting infectious viruses with high selectivity. The electrochemical sensing device introduced here (Apta-DEPSOR) for the quantitative monitoring of aptamer-target binding can generally be used for these purposes. This tool is sufficiently powerful, as shown in this study, and it has the merits of being portable and having a high cost performance (due to the disposable sensor chip used here[137]). In particular, it is favorable that its sensing part is composed of an aptamer that can be selected and evaluated by this device. Therefore, the application of this device for POC purposes (such as detecting the influenza virus at the spot of contagion) is very promising for use in the near future.

Chapter V Conclusion and Future Work

The competition-driven selection of DNA aptamers using multiple targets (termed as SELCO, Systemic Enrichment of Ligands by COmpetitive Selection) was first introduced in this study. The theoretical consideration of SELCOS revealed its potential difference relative to conventional SELEX. In particular, its methodological advantages will be reinforced by multiple target selection, with the simultaneous acquisition of multiple aptamers of high selectivity. SELCOS, which is PCR-free, has appropriate properties for wider categories of selection such as the *in vitro* selection of peptides/proteins. The experimental results confirmed our success in obtaining influenza virus subtype-selective aptamers using SELCO, which could be readily monitored with an electrochemical sensing tool (Apta-DEPSOR) as introduced here. By loading a selective aptamer as obtained (Apt03>T_{H1N1}) on its sensor unit, the feasibility of detecting the virus subtype was examined, and the detectability of subtype H1N1 ranged from 0.4 – 100 μg/mL. Although the situation in which the apta-DEPSOR can be useful is limited at present due to its sensitivity in the sub-μg/mL range, its portability (a merit of DEPSOR) enables us to collect important data at the POC (point of care).

Future studies about these topics are very exciting for the development of the SELCO field. Finally, we must note that developing SELCO solely for selecting DNA aptamers is, in principle, also applicable to other selection categories such as the *in vitro* selection of peptides/proteins[138,139] and the DNA-encoded library (DEL) selection of small molecules[140]. For these selections, the PCR-free nature of SELCO is very convenient because the troublesome retagging process (such as puromycin-linker ligation to mRNA) required for those technologies, can thus be discarded (SELEX is, conveniently, free from this tagging process).

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List of Publications

Journal Paper:

1. Competitive non-SELEX for the selective and rapid enrichment of DNA aptamers and its use in electrochemical aptasensor. Ankita Kushwaha, Yuzuru Takamura, Koichi Nishikagi and Manish Biyani (accepted in Scientific Reports).

Book Chapter:

2. Book title: Immunodiagnostic technologies from laboratory to point-of-care testing. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani, "Alternative analyte-binding compounds for immunosensor-like point-of-care application" (submitted)

Conference

1. Ankita Kushwaha, Yuzuru Takamura, Manish biyani; Multiplex Selection: On chip based inter-species selection of high affinity aptamers for multiple targets in parallel; *Bio-wakate Symposium*, Kanzawa (2016).
2. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Two-plex *in vitro* selection of DNA aptamer for multiple subtypes of Influenza A viruses, *The Electrochemical Society of Japan*, Toyama (2016).
3. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; AptaArray: Ultraspecific co-detection of multiple biomarkers using multiplex *in vitro* selection, *The 39th Annual Meeting of the Molecular Biology Society of Japan (MBSJ 2016)*, Yokohama (2016).
4. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Development of multiplexed in vitro selection of aptamers for multiplexed protein sensing, *JAIST Japan-India Symposium on Material Science*, Japan (2017).
5. Ankita Kushwaha, Yuzuru takamura, Manish Biyani; Ultra specific co-detection of multiple types of Influenza viruses using multiplex in vitro selection and electro-aptasensor; *5th International Conference on Bio-Sensing Technology*, Riva Del Garda-Italy (2017).
6. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Creation of smart aptameric reagents for the global antigenic diversity of Influenza viruses; *8th Annual Symposium-Bridging Nature and Technology ISAJ*, Tokyo (2017) (Oral Presentation)
7. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Highly selective and sensitive detection of emerging subtypes of Influenza viruses using SELCO and DEPSOR; *JAIST Japan-India Symposium on Material Science*, Japan (2018).

Award

1. Best Oral Presentation, in India-Japan Bilateral Conference on Impact in Make in India Efforts on September 22, 2015 in Biyani Girls College, India.
2. "JAIST President Award" in Japan Advanced Institute of Science and Technology on 9 March 2018.

Industrial Internship:

Industrial Internship in Fuji Electric, Malaysia with Industrial Attachment Program by University of Utari Malaysia (UUM) from 20 February-14 March 2017.